VOL. 26, NO. 2 AUGUST 1949

THE POISONING OF *MARINOGAMMARUS MARINUS* BY CUPRIC SULPHATE AND MERCURIC CHLORIDE

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(*Received* 20 *October* 1948)

(With Four Text-figures)

INTRODUCTION

Recently the toxic action of copper and mercury salts on various marine animals has been studied as part of the anti-fouling investigations conducted by the Marine Corrosion Sub-Committee of the British Iron and Steel Research Association (formerly of the Iron and Steel Institute). Harris (1946) has reported general toxicity results; Barnes & Stanbury (1948) have given results obtained with the Harpacticid *Nitocra spinipes* and discussed the physico-chemical state of copper and mercury salts in sea water, and Pyefinch & Mott (1948) have published detailed data on the sensitivity of barnacles and their larvae to the same poisons. The present paper is an account of work done on the amphipod crustacean, *Marinogammarus marinus* in the years 1946 and 1947 at the Millport Laboratory. In some respects the work is incomplete, but since these anti-fouling investigations are not being continued, it seems advisable to give a short account of the *M. marinus* results. *M. marinus* was used in these experiments since, although not a fouling organism, it is easily obtained in large numbers at all times of the year. It is a typical marine crustacean of whose physiology something, at least, is known; it lives well in the laboratory, and it survives for long periods in hypotonic sea waters and even for a number of days in distilled water. It is abundant in collections of gammarids from about mid-tide level at Millport and, by taking a constant size of animal from the collections, it is certain that only the desired species is used for experiments.

Concentrations of copper and mercury were determined by colorimetric methods, using a Spekker Photoelectric Absorptiometer. For copper estimations, the sodium diethyldithiocarbamate method (Harris, 1946) was used, and for mercury estimations, the dithizone method (Barnes, 1946; Barnes, 1947; Harris, 1946).

METHODS OF ASSESSMENT OF TOXICITY

Most studies of the poisoning of marine animals by metallic salts in solution have assessed toxicity in one of four ways. The first type of toxicity standard is based on the average time required to kill the organism when it is kept continuously in a solution having a definite concentration of the metal. The second is based on the average concentration of the poison required to kill the organism when it is only immersed for a definite period of time in the poison solution and then returned to its normal non-toxic environment. A third, which combines these two to some extent, is the toxicity assessment based on the average number of animals surviving a definite period of contact with a definite concentration of poison. Finally, there is the commonly used assessment—median lethal concentration—the concentration required to kill 50% of the animals within a definite period of time. All four methods have been used in anti-fouling researches; the third is that used by Pyefinch & Mott (1948) in work on barnacle larvae.

These methods all use an assessment of death and tend, in complex animals like crustacea, to give very variable results. For example, the Mean Lethal Times for batches of *M. marinus* immersed in sea water with concentrations of mercury ranging from 10 to 50 mg./l. vary from 28 to 5 hr. The standard error of the mean, in batches of fifty animals at each concentration, is of the order of 26% of the Mean Lethal Times of each five animals determined separately. This obviates all but the coarsest use of this measure of toxicity.

Toxicity has, however, been assessed by methods not based on killing; for example the work of Cole (1932) on the rate of cirral beat in barnacles, and the extensive work done on the effects of various ions in solution on the rate of heart-beat in such crustacea as *Daphnia* spp.

The extent of activity of an organism may be used to assess toxicity. *Gammarus* spp. from fresh, salt and estuarine waters all show a progressive reduction in activity in solutions of copper and mercury salts (unpublished results of Mr K. A. Pyefinch and the author). With *Marinogammarus marinus*, five activity ratings were used: full activity (rating 5), moderate activity (rating 4), only pleopods active (rating 2), only isolated appendages active (rating 1) and cessation of all movement (death, rating 0). All experiments were carried out in Petri or similar flat dishes where observations on level of activity could be made with the minimum of disturbance. Table 1 shows the average time which animals spent in each category during poisoning with three concentrations of mercury in sea water.

Table 1. *Periods in activity ratings*

		Average time taken between each rating (in min.)			
Activity states ...		G.A.–M.A.	M.A.–P.A.	P.A.–I.A.	I.A.–Q.
Ratings ...		5–4	4–2	2–1	1–0
Concentration of Hg. (mg./l.):	50	27	63	35	40
	30	59	118	67	75
	10	168	407	193	223

These averages (drawn each from twenty-five separate animals) show that the drops from general activity (G.A.) to moderate activity (M.A.); from plepod activity (P.A.) to activity of isolated appendages (I.A.), and from activity of I.A. to quiescence (Q.), are of approximately equal duration. The drop from M.A. to P.A. covers a period approximately twice as long. This justifies the ratings of 5, 4, 2, 1 and 0 adopted. The only possible exception is that the drop from I.A. to Q. apparently covers a longer period than the drops from G.A. to M.A. and from P.A. to I.A. If these assessments of activity are plotted against time 'activity curves' result. From these curves, the time value can be read off which corresponds to the mean of the total score for the activity ratings of a number of animals (e.g. if ten animals per experiment are used, the time value corresponding to an activity assessment of twenty-five is read off). This time value is termed 'Mean Activity Time' (M.A.T.). Using M.A.T. as a measure eliminates the more variable part of the 'activity curves'. Also, it was noted (see Table 1) that the drop in activity from I.A. to Q. apparently covers a longer period than the drops from G.A. to M.A. and from P.A. to I.A. However, this stage (I.A. to Q.) is usually only reached by individuals when the group of animals as a whole has suffered quite considerable reduction in activity (i.e. after the M.A.T. has been passed). Therefore the effect of this discrepancy is nullified if M.A.T. is used.

M.A.T.'s show less variation than Mean Lethal Times (M.L.T.). For example, M.A.T. was calculated separately for each of ten batches of ten *M. marinus* immersed in sea water with concentrations of mercury from 10 to 50 mg./l. Although the M.A.T.'s ranged from 9 hr. to 90 min. over the series of concentrations studied, the standard deviation of the M.A.T. at each concentration is much lower than the corresponding deviation in M.L.T.'s. With 50 mg./l. mercury in sea water this standard deviation is 6·25 % of the value of the mean of the M.A.T.'s. The standard deviation becomes lower with lower concentrations of poison (i.e. at lower M.A.T.'s).

Whatever statistical importance is attached to the M.A.T., it is at least a reliable and representative measure of the effect of a given concentration of poison on a given population. It is certainly more satisfactory than any measure which requires the determination of 'death'.

TOXICITY AND 'MEAN ACTIVITY TIME'

Toxicity experiments were carried out in Petri dishes which were filled with a standard volume (usually 60 ml.) of poison solution, after experiments had shown that the total amount of poison solution might have an effect on poisoning. These volume experiments will be discussed later.

When M.A.T. is plotted against concentration of copper or mercury, characteristic 'toxicity curves' result. Fig. 1 shows the effect of mercury; in both media, distilled water and sea water, the result is an 'L-shaped' curve. It can be seen that in the lower concentrations of poison there is a range (vertical arms of the curves) over which a slight decrease in concentration will bring about a very great increase in the time required for poisoning. Over a range of higher concentrations (horizontal arms of the curves) a large decrease in concentration will only bring about a slight increase in the time required for poisoning. Similar toxicity to concentration relations have been described by Jones (1940, 1941*b*), who terms the change of relation between toxicity and concentration the 'threshold of toxicity'.

It should be pointed out that the 'vertical arms' of the curves are not truly vertical (i.e. there is not a critical concentration detectable below which mercury has no toxic effect). For example, the M.A.T. of *M. marinus* in 5 mg./l. mercury in sea water is 85 hr. With the present technique it is impossible to tell if mercury has

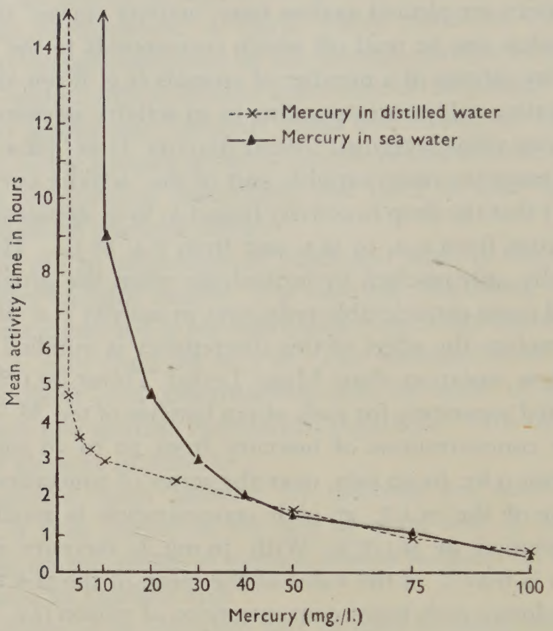

Fig. 1. Poisoning of *M. marinus* by mercury; the relation between M.A.T. and concentration of poison.

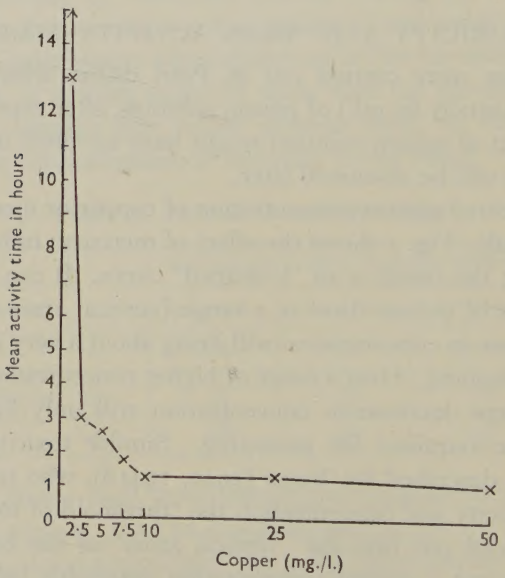

Fig. 2. Poisoning of *M. marinus* by copper; the relation between M.A.T. and concentration of poison.

an effect at concentrations of less than 2·5 mg./l. in sea water and 0·5 mg./l. in distilled water. Controls at such periods tend to decrease in activity due to lack of food and other unnatural experimental conditions. It does seem likely that the concentration at which mercury ceases to have a toxic effect is minute. The significant difference between the curves for animals in distilled water and in sea water lies in the shifting of the turning point of the curve—that is in the alteration of the concentration beyond which the M.A.T. is not greatly affected by further increases in concentration.

The effect of copper in distilled water is (as shown by Fig. 2) similar to that of mercury. Again there is an 'L-shaped toxicity curve' with a turning point (i.e. a concentration beyond which the M.A.T. is not greatly affected by further increases in concentration) which is markedly similar to the turning point of the curve for mercury in distilled water.

The effect of copper in sea water is difficult to study. Using cupric sulphate as the source of copper, if the concentration of this element in sea water exceeds 7 mg./l., precipitation of basic copper salts occurs. Up to this concentration *M. marinus* is completely insensitive to copper. However, solutions with copper concentrations of up to and more than 100 mg./l. can be obtained in an artificial sea water (consisting of the chlorides and sulphates of sodium, magnesium, calcium and potassium). Pyefinch & Mott (1948) discuss the use of this artificial sea water. The effect of copper on *M. marinus* in this medium was studied, and it was found that copper showed no markedly toxic effect in concentrations of less than 50 mg./l. That these results can be used to compare with results for mercury in sea water is demonstrated by some other facts. In mercury poisoning in this medium, the effects of various concentrations are exactly the same as those in natural sea water. The oxygen uptake of *M. marinus* is the same in both natural and artificial sea water.

Unlike the case of mercury, which in distilled water shows a markedly similar toxic range to that shown in sea water, copper in distilled water shows a toxic range differing widely from that in sea water.

Experiments showed that alteration of the volume of solution (in the case of copper in distilled water) had a pronounced effect on the M.A.T.

Table 2. *Mean Activity Times in various volumes of poison solutions*

		Volume of solution of copper in distilled water			
		500 ml.	200 ml.	75 ml.	25 ml.
Concentration of Cu (mg./l.):	0·25	5 hr. (125)	?12 hr. (50)	> 100 hr. (18·75)	> 100 hr. (6·25)
	0·5	3 hr. (250)	3 hr. (100)	> 100 hr. (37·5)	> 100 hr. (12·5)
	0·75	4½ hr. (375)	3 hr. (150)	? hr. (56·25)	> 100 hr. (18·75)

(Figures in brackets represent the total *amount* of copper available at each concentration and volume, expressed in micrograms.)

Concentrations, which are distinctly subtoxic in smaller volumes, become rapidly toxic in larger volumes of poison solution. There appears to be a critical level in the total amount of copper available. In these experiments, with five animals in each volume of poison solution, it is of the order of 50 μg. of copper.

It was thought that the cause of this critical level might be that up to 10 μg. copper per animal is adsorbed on to the animal's cuticle; that is, copper is only available for poisoning after these surface requirements have been satisfied. Accordingly, experiments were carried out in which *Gammarus* were given a preliminary dip (duration 2 sec.) in a relatively high concentration of copper (e.g. 100 mg./l.) and then removed to a solution of lower concentration. After this preliminary immersion, low concentrations proved more rapidly toxic (Table 3). It should be noted that the preliminary immersion also affected those control animals which were afterwards returned to distilled water. It was also found by analysis of the dipping solutions that the amount of copper removed during 2 sec. dips in various copper concentrations was small and very variable.

Table 3. *Effect of preliminary immersion in* 100 *mg./l. copper on mean activity times*

	Concentration of copper in distilled water (mg./l.)							
	50	5	0·75	0·5	0·25	0·1	0·05	0 (control)
With preliminary immersion	30	30	36	42	45	48	60	84
Without prelim. immersion	60	150	900	> 1200	> 1200	> 1200	> 1200	> 1200

(Mean activity times in minutes)

Another possible explanation of the results is that at one stage in the entry of copper to the animal, the metal has to pass a membrane whose permeability to copper varies with the concentration of that element in the external medium. In such a case, the effect of immersion in a very high concentration of copper might be to allow a toxic amount of copper to enter in a comparatively short time interval (i.e. in preliminary immersion in 100 mg./l. copper, during the 2 sec. dip). The membrane could be the normal chitin-cuticle body wall, the modified bounding membrane of the gills, or the mid-gut wall. In the last case, that of entry through the gut wall, the theory could be advanced that the preliminary immersion solution constitutes such an unnatural medium that the animals invariably 'gulp' solution into their gut during the short period of immersion.

The volume of solution has an effect also in poisoning by mercury in distilled water (Table 4).

Table 4. *Mean activity times in various volumes of poison solutions*

		Volume of solution of mercury in distilled water	
		500 ml.	25 ml.
Concentration of Hg (mg./l.):	1.0	390 min.	> 40 hr.
	0·5	450 min.	> 40 hr.
	0·1	510 min.	> 40 hr.

Again, concentrations which, when a volume of 60 ml. is used, are subtoxic, prove toxic within a relatively short time when a volume of poison solution of 500 ml. is used. From further experiments, however, there was no very apparent 'critical level' in the total amount of available mercury necessary for poisoning. There is no critical 'step' in the total amount (corresponding to the 10 μg. in copper poisoning) above which toxicity increases rapidly.

It may be the case that mercury acts directly as a simple poison, and it is perhaps cumulative in nature. Copper may, on the other hand, be toxic in more than one way. For example, it seems possible that some effect due to the presence of small amounts of this element may occur when the animals are in a disadvantageous environment (i.e. in large volumes of exceedingly hypotonic solutions), and an effect of this kind is possibly distinct from that exerted when larger amounts of copper are available.

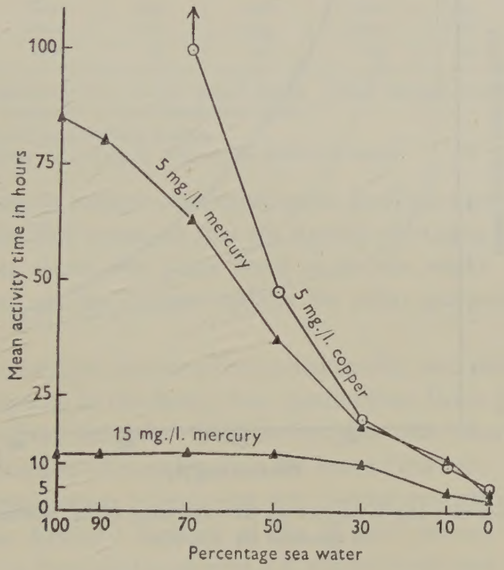

Fig. 3. The effect of salinity on the poisoning of *M. marinus* by copper and mercury; the relation between M.A.T. and percentage sea water at certain concentrations of poison.

To examine in more detail the differences between poisoning in sea water and in distilled water, toxicity experiments were carried out at a number of concentrations of copper and mercury in mixtures of various salinities. Fig. 3 shows the results for 5 and 15 mg./l. mercury, and for 5 mg./l. copper. The curve for 5 mg./l. copper is typical of those for that metal. Here, poisoning only becomes apparent below 70% salinity, and toxicity is rapidly increased by any decrease in salinity. The curve for 15 mg./l. mercury represents the typical effect of decrease in salinity on poisoning by that metal. The curve for 5 mg./l. mercury illustrates the maximum effect of salinity change. This latter concentration (see Fig. 1.) in sea water lies in the range (vertical arm of the curve), where a slight increase in concentration produces a great decrease in M.A.T., and in distilled water lies in the range (horizontal arm of the curve) where a large increase in concentration only produces a slight decrease in M.A.T.

The effect of mixtures of copper and mercury was studied. The most interesting results were obtained when traces (well below the toxic range) of one metal were added to a range of concentrations of the other. Low concentrations of copper (0·2, 0·5, 1 and 2·5 mg./l.) increased appreciably the toxicity of a series of mercury solutions in sea water and in distilled water. The effect of 1 mg./l. copper on the toxicity of a range of mercury concentrations in sea water is shown in Fig. 4. The effect of the small amount of copper is much more than additive and the 'toxicity curve', while keeping essentially the same form, is displaced in a similar fashion to that displacement resulting from a decrease in salinity. On the other hand, similarly small concentrations of mercury (0·4 and 0·75 mg./l.) do not affect the toxicities of a wide range of copper concentrations in both natural and artificial sea water and in distilled water.

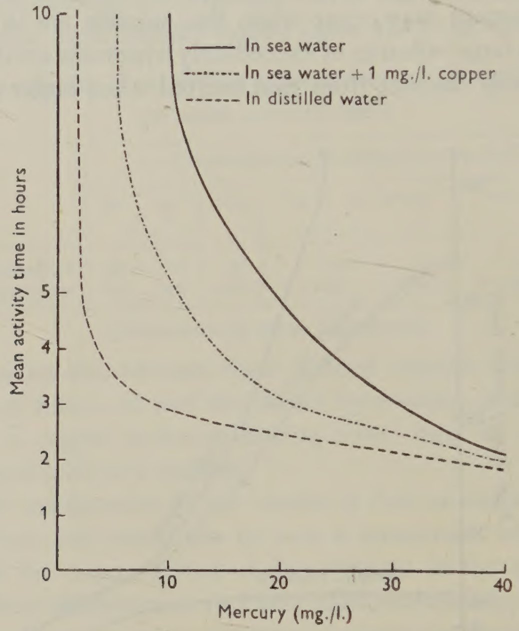

Fig. 4. The effect of 1 mg./l. copper on the poisoning of *M. marinus* by mercury as assessed by M.A.T.'s.

The physico-chemical and biological aspects of a similar synergic effect are examined by Barnes & Stanbury (1948).

OXYGEN CONSUMPTION AND RATE OF HEART-BEAT

The oxygen consumption of animals under various conditions was found by small-scale Winkler estimations of dissolved oxygen. Most attention was paid to the effect of copper and mercury in the earliest stages of poisoning (i.e. before visible symptoms of poisoning appeared). It is important to distinguish here, as far as possible, between an initial lowering of oxygen consumption as an immediate symptom of poisoning, and a lowering of oxygen consumption as a final result of the cessation of metabolism in poisoned tissue. The respiration results were drawn only from animals which showed no decrease in activity through the experiments, and it is assumed that only the former depression of oxygen uptake was demonstrated since only comparative figures were required. The calculation of results was simplified; the results of Table 5 are calculated for a standard weight of animals in a respiratory chamber of standard volume at a constant temperature, and they are related to the normal oxygen consumption of *M. marinus*.

Table 5. *Comparative oxygen consumption*

Period (min.)	5	10	15	20	25	30	35	40
A	100	100	100	100	100	100	100	100
B	116	100	110	135	127	116	117	113
C	118	23	17	29	23	29	—	—

A: Normal oxygen consumption in sea water (100). (Also oxygen consumption in artificial sea water, and in 20 mg./l. mercury in sea water: see text.)
B: Oxygen consumption in distilled water.
C: Oxygen consumption in 25 mg./l. copper in distilled water.

There is a small rise in oxygen consumption in distilled water. There is no change in activity state in distilled water, so that the uptake of oxygen for active movements probably remains at about the same level as in sea water. The small increase represents, therefore, all the oxygen uptake for other purposes, for example, for osmo-regulation.

Copper lowers the oxygen consumption considerably and mercury not at all. The increase in oxygen uptake in the first 5 min. period (see Table 5) is due to the initial increase in activity of the animals. Such an increase was noted in *Gammarus pulex* on immersion in solutions of metallic ions by Jones (1941*a*).

Another type of respiration experiment was carried out wherein respiratory rates were determined for different sections of the 40 min. period covered by Table 5. Animals were set up in Petri dishes as in M.A.T. experiments and after varying periods transferred to the respiration chamber where their rate of oxygen consumption was determined over a 20 min. period. Artificial sea water was used instead of natural sea water, thus allowing higher concentrations of copper to be used.

The averages of a number of results are shown in Table 6 (all results ± 4% which is the variation of control experiments).

Table 6. *Percentage lowering of oxygen consumption*

Medium	Concentration of poison	Period of poisoning over which oxygen consumption calculated (%)		
		0–20 min.	10–30 min.	20–40 min.
Distilled water	25 mg./l. Cu	63	57	60
	50 mg./l. Hg	—	0·5	4
Artificial sea water	25 mg./l. Cu	28·5	31·5	36
	50 mg./l. Hg	4	3	5

Copper lowers the oxygen consumption considerably and mercury not at all, also copper apparently lowers the oxygen consumption more in distilled water. During all these experiments the animals were all active at the end of the experiment (i.e. there were no grossly visible symptoms of poisoning).

Increase in the concentration of copper tends, within limits, to cause a further decrease in oxygen uptake. There is an indication that the effect of copper on oxygen uptake is greater at lower oxygen tensions. This was investigated by a parallel series of experiments in which the determinations of oxygen consumption were made over double the periods of Table 6. The average lowering of oxygen consumption for this type of experiment is 75 % over the first 40 min. of poisoning in 25 mg./l. copper as against the average lowering of oxygen consumption of 60% in normal experiments. During the longer experiments the oxygen tension of the control animals dropped from 10·4 to 5·5 mg./l. dissolved O_2 as against a drop from 10·4 to 7·9 in normal experiments.

It was thought that an investigation of the heart-beat rate in copper and mercury solutions might throw some light on the penetration of the poison ions. The heart-beat of *M. marinus* is spasmodic. Three measures were adopted: the overall rate, over a period of approximately a minute or longer; the spasmodic rate or the rate during the spasms of beating; and a measure of the degree of spasmodicity calculated from the first two rates. Generally the effects of all toxic solutions are similar:

(1) An initially higher overall rate of heart-beat tends to return to normal as poisoning progresses.

(2) The spasmodic heart-beat rate is greatly increased over the normal and remains nearly constant or is increased slightly as poisoning progresses.

(3) The degree of spasmodicity of heart-beat follows a very constant variation during the course of poisoning. Initially it drops to a value well below that of normal animals, then increases steadily during poisoning, eventually reaching a value far in excess of the normal.

DISCUSSION

It is seen that the toxicities of copper and mercury to *Marinogammarus marinus* differ widely and that the differences are not only those of scale.

The toxicity of mercury apparently varies according to a fairly simple relationship with the concentration of the metal. The toxicity in sea water over the range of concentrations from 2·5 to 20 mg./l. increases rapidly with small increases in concentration, but in higher concentrations does not increase greatly even with large increases in concentration. The toxicity of mercury in distilled water conforms to the same relationship (see Fig. 1).

On the other hand, copper in sea water (i.e. high concentrations in artificial sea water) has virtually no toxic action on this species in concentrations of less than 50 mg./l. In distilled water, however, low concentrations (0·5 mg./l.) prove toxic and the toxicity of copper, in this case, seems to bear much the same relationship to concentration of the metal as is the case in mercury poisoning (see Fig. 2).

Some light is perhaps thrown on this by the results of the oxygen uptake experiments. The rate of oxygen consumption is depressed by copper but not by mercury.

Therefore it is suggested that in *M. marinus* mercury acts by direct and general poisoning of the protoplasm. Its toxic action is not greatly affected by other conditions, for example, decrease in salinity. It is toxic at very low concentrations which, however, require a proportionately long exposure period.

On the other hand, copper probably acts less directly by its action on the equilibrium of some physiological system. It lowers the rate of respiration but is virtually non-toxic when all other conditions are optimal. It becomes more toxic on decrease in salinity and perhaps on lowering of oxygen tension in the medium. It has a pronounced effect, in quite small quantity, on animals being poisoned by mercury. The toxic action of copper in *M. marinus* appears to be basically its interference with either the respiratory system or the osmo-regulatory system of the animal, or both.

In conclusion, it can be stated that the toxicities of copper and mercury to *M. marinus* are very dissimilar, not only in degree, but also in kind (Hunter, 1947). The simple poisoning of mercury has its action not greatly altered by changes in external conditions, while the indirect poisoning of copper is greatly affected by, if not due to, such changes.

American workers on the anti-fouling problem have published results supporting the view that copper retards vital processes through inactivation of essential enzymes (e.g. Clarke, 1947 on barnacles and mussels; Miller, 1946 on *Bugula neritina*).

Many workers have proposed that there is a common mechanism in the action of heavy metal salts on all organisms. Jones (1940, 1941 *a*) discusses most theories. In the course of the anti-fouling investigations studies on the poisoning of many marine organisms by copper and mercury salts have been carried out. It has been apparent that the resistances of various organisms to any particular concentration of poison in their environment differ widely (Harris, 1946). Differences occur between closely related species (see Pyefinch & Mott, 1948). It is therefore impossible to attempt to deduce a common mechanism in poisoning by heavy metals in sea water.

The following might be put forward as a hypothesis about the action of heavy metals on complex animals. In any single species there is a chance of poisoning and death resulting from a number of different causes, dependent on the concentration of the metal. The effects which have been noted by many workers may form an overlapping series; for example, at a very low concentration the only effect may be stimulatory, at a slightly higher concentration an enzyme system might be inactivated, at a higher concentration the metal might interfere with respiration by decreasing the blood pigment's transport efficiency or interfere with excretion or nervous co-ordination, at a higher concentration still the metal might coagulate mucus or other essential secretions, while at very high concentrations coagulation of the protoplasm itself, or fixation, will take place.

SUMMARY

The toxicity, under various conditions, of cupric sulphate and mercuric chloride to the marine amphipod, *Marinogammarus marinus*, has been studied. With optimum conditions copper is virtually non-toxic over the range of concentrations studied (e.g. becoming effective as a poison on decrease in salinity or oxygen tension). The toxic action of mercury is not greatly affected by changed conditions. At very low concentrations, copper has a pronounced effect on animals undergoing mercury poisoning. Subtoxic traces of mercury have no effect on the course of copper poisoning. It is suggested that in *M. marinus*, mercury acts directly by poisoning of the protoplasm while the effect of copper is indirect, involving the upset of some metabolic process.

The relation of this work to other studies of the action of heavy metals is briefly discussed.

I wish to record my thanks to the Marine Corrosion Sub-Committee for their permission to publish this paper, and to Prof. J. E. Harris for his encouragement at all stages of the work. I am also deeply indebted to Mr K. A. Pyefinch, to Dr H. Barnes, and to the late Director, Mr R. Elmhirst, and the Staff of the Marine Station, Millport, for all their assistance.

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THE PIGMENTATION OF CAVERNICOLOUS ANIMALS

III. THE CAROTENOID PIGMENTS OF SOME AMPHIPOD CRUSTACEA

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(*Received* 11 *November* 1948)

INTRODUCTION

During an investigation of the causes of pigment lack in cave Crustacea, it seemed desirable to study the kind of pigmentation in related above-ground (epigean) forms. The colour of three species of Isopoda was found to be due to a melanin-like pigment in branched cells (Baldwin & Beatty, 1941). Two plant carotenoid pigments—cryptoxanthine and β-carotene—were present throughout the body tissues of one (*Asellus aquaticus*), but did not contribute significantly to the general colour of the animals. In the present work, it has been demonstrated that the colour of three species of Amphipoda is due wholly to carotenoid pigments, including in each case a proportion of a specific animal astacin-like pigment.

TECHNIQUE

For separation of the carotenoid pigments the standard methods have been used as described by Zechmeister (1934) and Zechmeister & Cholnoky (1938). The petrol ether used throughout was of the 50–60° boiling-point grade. The magnesium oxide used for adsorption was the arsenic-free product of British Drug Houses. Adsorption columns were about 1 cm. in width and 10 cm. in height. Absorption maxima were determined with a Hartridge Reversion Spectroscope with a symmetrical slit and greatest probable error of $\pm 2\mu\mu$.

EXPERIMENTAL

Preliminary investigations have been made of the pigments of three amphipod species, *Gammarus pulex* L., *G. marinus* Leach and *Orchestia gammarella* Pallas. From these species the pigment can be extracted with organic solvents, leaving the bodies entirely decolorized except for the eyes, which remain black. The externally visible coloration of three Isopoda species studied (Baldwin & Beatty, 1941) is due to a melanin, but melanin is absent from the amphipod species unless, perhaps, the eye pigment is a member of this group. In all our observations, typical carotenoid behaviour was exhibited by the pigments extracted, and the animals would therefore appear to owe their visible coloration to carotenoid material almost if not quite exclusively.

A total of 2·9 g. of *Gammarus pulex* were extracted with acetone and the pigment transferred to petrol ether and partitioned against 90% methanol. More hypo- than epiphasic pigment was present. The epiphasic materials were examined and the hypophasic fraction rejected, to be studied again later on a separate batch of animals. The petrol-ether solution was chromatographed on calcium carbonate and gave a deep, reddish brown band at the top of the column. This was developed with 2% methanol in petrol ether and gave rise to a pale pink band in the lower part of the column with a small, pale yellow band below it.

The upper band was eluted and found to be still wholly epiphasic; it was saponified in the usual way, recovered in petrol ether and again submitted to the phase test. Very little pigment was now epiphasic, but the material that passed into the petrol ether at this stage remained epiphasic in the presence of traces of alkali and of acid and was therefore probably a carotene. The material which was now hypophasic became epiphasic again in the presence of traces of acid but passed back into the alcoholic phase in the presence of a trace of alkali. It was found to exhibit a single absorption maximum at 498 $\mu\mu$ in carbon disulphide (cf. the value of 500 $\mu\mu$ found for lobster astacin in this solvent by Kuhn & Lederer, 1933). This behaviour is typical of a group of compounds, the so-called carotenoid acids, of which a number have been investigated by various workers (see, for example, the list given by Strain, 1938). These compounds are particularly interesting in that except for a few yeasts and unicellular algae they are not known to occur in plant materials, but appear to be specifically elaborated by the animal organism.

A further sample of *G. pulex* was extracted, and without previous partitioning or saponification the whole pigment was chromatographed in petrol ether on magnesium oxide. A strong purple band appeared at the top of the column and was developed with 0·5% methanol in petrol ether. A small yellow band, which probably represents the carotenoid acid and other epiphasic material described in the previous paragraph, separated from the main band and was eluted through the column and discarded. The development was continued with 1·5% methanol in petrol ether and four bands were obtained in the following order from top to bottom: band 1, pink; band 2, yellow; band 3, pale pink, extensive but containing very little pigment; band 4, rather deep yellow in colour. These were eluted separately and each was found to be wholly hypophasic. After saponification they remained hypophasic in the presence of acids and alkalis and therefore belonged to the xanthophyll group rather than to that of carotenoid acids. The absorption maxima of band 2 after saponification were 506 and 473 $\mu\mu$ in carbon disulphide, but these values approximate to those of a number of xanthophylls and the pigment could not be identified further. The material from band 4 showed maximal absorption at 510 and 476 $\mu\mu$ in carbon disulphide (lutein?). Bands 1 and 3 contained too little pigment for further examination.

The carotenoid pigments of *G. pulex* therefore include a carotene (probably), and at least four xanthophylls, of which two are present in considerable quantity, and one is perhaps lutein. These are accompanied by a carotenoid acid.

The detection of this carotenoid acid among the products of saponification of those pigments which were epiphasic in the first partition suggested that the original material contained an epiphasic ester of the acid in question. Sörensen (1936) had previously reported the presence in the same species of a hypophasic ester of some carotenoid acid which he considered to be astacin, but in the present work there was no indication of any ester apart from the epiphasic material already mentioned.

There is considerable confusion in the literature on the subject of carotenoid acids, especially in the case of the free and esterified forms of the only one which has been exactly identified and of which the constitution has been definitely established, namely, astacin, a tetra-keto-β-carotene. Kuhn & Lederer (1933) reported that an epiphasic ester of astacin can be extracted from the scarlet hypodermis of *Astacus gammarus* and a hypophasic ester from the eggs of the same species, both substances being readily transformed into astacin by saponification. The subsequent work of Kuhn & Sörensen (1938) has, however, made it clear that the 'hypophasic ester' is in reality not an ester at all, but a dihydroxydiketo-β-carotene to which the name astaxanthine was given. This substance is freely autoxidizable to yield astacin itself in alkaline solutions such as are commonly used for the saponification of xanthophyll esters. The epiphasic ester is probably not an ester of astacin, therefore, but of astaxanthine, astacin itself being an artefact.

Comparing the present results with those of Sörensen it may be suggested that the epiphasic material was an ester of astaxanthine, and that the hypophasic material described by Sörensen was either free astaxanthine (which is very improbable) or else astacin derived from it in the course of his manipulations which, unfortunately, are not described in any detail in his paper. For present purposes, however, the important point seems to us to be the demonstration of the presence of a carotenoid acid, or of some ester of such an acid, in the tissues of *Gammarus pulex*. Further work on much larger quantities of material will be necessary for its exact identification.

Turning now to the second species examined, several hundred *G. marinus*, weighing about 5 g., were collected from the shore at Heacham, Norfolk. They were extracted with methanol in the usual way, becoming completely decolorized in the process apart from the eyes, which remained black as before. The fine orange-yellow extract was treated with petrol ether and water and the pigments quantitatively transferred to petrol ether and partitioned against 90% methanol as usual. More pigment was hypo- than epiphasic.

The epiphasic fraction was washed with 90% methanol until no more colour was extracted, and the remaining wholly epiphasic material chromatographed on a column of magnesium oxide from solution in petrol ether. A large pink band appeared in the upper part of the column and gave rise on elution to a pigment which showed maximum absorption at 495 $\mu\mu$ in petrol ether. Below it was a smaller brownish red band, the pigment of which showed an absorption maximum at 490 $\mu\mu$ in petrol ether. After saponification the pigment of the lower band was almost wholly hypophasic in the presence of alkali but epiphasic in the presence of traces of acid. The carotenoid pigments of *G. marinus*, like those of *G. pulex*, thus include a carotenoid acid, again in the form of an epiphasic derivative.

Seventy-three large specimens of *Orchestia gammarella*, weighing 2·7 g., were quietened with chloroform vapour and extracted with methanol. Again the animals were quite decolorized apart from the eyes, which remained black. The pigments were found to be wholly hypophasic, and after recovery in petrol ether were chromatographed on calcium carbonate and the chromatogram developed with petrol ether. Four well-separated bands rapidly appeared, and of these, three were rather small and pale yellow in colour. These were discarded. The remaining band, the third from the top of the column, was large and orange-yellow in colour, and was eluted and saponified. It was then found that the pigment was wholly hypophasic in presence of alkali, whereas in presence of a trace of acid a small part became epiphasic. It may be concluded that some xanthophyll was present together with a little carotenoid acid.

We may conclude therefore that the external colour of *Gammarus pulex*, *G. marinus* and of *Orchestia gammarella* is wholly due to carotenoid pigments. Other pigments are present in traces at most, and the black colouring matter of the eyes is perhaps a melanin. In contrast to the Isopoda (Baldwin & Beatty, 1941), all three Amphipoda gave evidence of containing acidic carotenoid material, i.e. they contain endogenous as well as exogenous carotenoid pigments.

PIGMENTS IN CAVERNICOLOUS AMPHIPODA

The Amphipoda include many cave types, as may be seen in the summary in Spandl (1926), the majority being either white or colourless. Specimens of the large cave species *Niphargus* (*Stygodytes*) *balcanicus* Absolon and another large *Niphargus* species from caves in the Popovopolje, Herzegovina (Jugoslavia), were examined. (I am indebted to Dr. I. Gordon for advice that the second species corresponds as far as is known to the still incompletely described *Niphargus* (*Antroplotes*) *herculeanus* Absolon.) They were whitish in appearance and yielded no trace of pigment to the alcohol in which they were preserved. Some specimens of a small *Niphargus* species from the Postumia Grotte, north Italy, were extracted with methanol, and any possible pigment transferred to a very small volume of petrol ether, but no trace of coloration could be observed.

In the case of *Asellus* it is evident that the cave form has lost melanin, for traces of the typical branched melanin-bearing cells are to be seen in the integument even of very pale specimens. Among the Amphipoda, however, the absence of pigment is complete, and it is difficult to determine what was the ancestral pigment. From our observations on epigean Amphipoda it is to be noted that three species, chosen at random, all had a wholly carotenoid pigmentation, including in each case a carotenoid of specifically animal type. A much greater range of species and genera will have to be investigated before it will be possible to state definitely whether carotenoid pigments are or are not of general occurrence in the Amphipoda. It is a matter of general experience, however, that amphipod species have a greenish, yellowish, or red pigmentation, which is rapidly removed when the specimens are preserved in alcohol, and it is to be expected that they contain carotenoid pigments. Details of the colouring and pigmentation of some epigean and hypogean isopods and amphipods are given in Table 1.

The nature of amphipod pigmentation is of special interest in that there is a certain amount of evidence that it is affected by light. Viré (1900), quoted by

Table 1. *Colourings and pigmentation of epigean and hypogean Isopoda and Amphipoda*

Data for epigean species, *Asellus aquaticus cavernicolus* and *Niphargus* from Baldwin & Beatty (1941) and present paper. Data for remaining species from Chappuis (1927).

Name of animal	External colour	Pigment responsible for external colour
EPIGEAN ISOPODA:		
Asellus aquaticus, A. meridianus	Yellow-brown to black	Melanin
HYPOGEAN ISOPODA:		
Asellus aquaticus cavernicolus	White to yellow-brown	None, or melanin
Johannella purpurea	Wine-red	
Anina lacustris	White if hypogean, ash-grey if epigean	
Asellus gauthieri, Janirella pusilla, Phreatoicus assimilis, P. typicus, P. kirkii, P. latipes, Phreatoicoides gracilis, Cruregens fontanus, fam. Cirolanidae, nearly all species, Titanethes, several species	None	
EPIGEAN AMPHIPODA:		
Gammarus pulex	Green-grey in general	Carotenoid acid + carotenoids
Orchestia gammarella	Olive-grey in general	Carotenoid acid + carotenoids
Gammarus marinus	Green-grey in general	Carotenoid acid + carotenoids
HYPOGEAN AMPHIPODA:		
Niphargus, numerous species	None, usually, but pink eggs in *Niphargus balcanicus*	No trace of carotenoids in 3 species
Niphargopsis legeri, Neoniphargus westralis, Metohia carinata, Phreatogammarus fragilis, Hyale jeanneli, Hyale incerta	None	

Verne (1926), showed that *Gammarus puteanus* lost its colour when kept in darkness for 20 days. According to the same author (Viré, 1904), this is also true for *G. fluviatilis* kept in the dark for 6 months. Viré stated (as quoted by Verne, 1926) that the colourless cavernicolous *Niphargus puteanus* developed a greenish brown colour after 2 months' exposure to light, but he found no such effect in *Niphargus plateaui* (Viré, 1900, 1904). Meek (1929) has made the interesting observation that specimens of the epigean form *Gammarus duebeni* living underground in the Mill Pit at Blyth are a 'dull transparent white', but that after exposure to light they develop the normal colour of the species, including the three characteristic red spots.

It would thus appear that light may be necessary for the development of the carotenoid pigmentation of Amphipoda, and that the darkness of the cave environment may provide an explanation of lack of pigment in cavernicolous Amphipoda. The possibility that scarcity of carotenoid pigment in the food of cave Crustacea accounts for lack of pigmentation in the animals has already been ruled out, for the animals are unpigmented whether or not they have access to carotenoid material in their food (Beatty, 1941).

SUMMARY

1. The visible coloration of three species of amphipod Crustacea (*Gammarus pulex*, *G. marinus* and *Orchestia gammarella*) was found to be due almost exclusively to carotenoid pigments, including in each case a proportion of an astacin-like carotenoid acid.

2. No carotenoid pigments could be demonstrated, even in traces, in three species of the cavernicolous Amphipod genus *Niphargus*.

3. Carotenoid pigmentation is thought to be characteristic of Amphipoda and would therefore be the ancestral pigment of the cave forms. The lack of pigment in cave Amphipoda may be due to absence of light; it is not due to lack of carotenoid pigments in the food.

This work was carried out in the Zoological and Biochemical Laboratories, Cambridge, during the tenure of a maintenance grant from the Department of Scientific and Industrial Research; it was supervised by Dr Ernest Baldwin, of the Biochemical Department, Cambridge. I wish to express my thanks for facilities offered me by Prof. J. Hadži and Prof. R. Kenk of the Univerza Kralje Aleksandra I, Ljubljana, and by the Director and Staff of the Oceanografski Institut, Split, Dalmatia.

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THE ABSORPTION OF WATER BY THE EGGS OF *CORIXA PUNCTATA* ILLIG. (HEMIPTERA-CORIXIDAE) UNDER EXPERIMENTAL CONDITIONS

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(*Received* 12 *November* 1948)

(With Two Text-figures)

Poisson (1924) states that the eggs of aquatic Hemiptera increase in volume as they develop. Kerenski (1930), Ludwig (1932), Evans (1933), Johnson (1937), Roonwal (1936), and Birch & Andrewartha (1942), all have described in various insects the changes which take place in the developing egg and which are generally associated with the absorption of water.

The eggs of Corixidae are aquatic and are normally in contact with water throughout their life. Poisson regards salinity of the external medium as an important factor affecting the distribution of adult aquatic Hemiptera. *Corixa punctata* Illig. is a species which occurs normally in fresh water, but is sometimes found associated with typically euryhaline species in brackish water.

It was thought that factors such as salinity and temperature, which might affect the uptake of water by the egg, might influence significantly the distribution of the species. Experiments were carried out, therefore, to investigate the changes in water uptake by the eggs of *C. punctata* under various conditions of temperature and salinity.

The nomenclature adopted in this paper is that given by China (1943).

METHODS

Female insects were collected from three localities in south Hampshire during the springs and summers of 1938 and 1939, and kept in separate aquaria in the laboratory. Eggs were laid on weed, usually overnight, and were collected daily. A newly captured female laid sufficient eggs at a time to provide a batch suitable for weighing (about six to fifteen eggs). As a rule, a bug laid increasingly fewer eggs each day. Eggs were examined, washed clean and put into distilled water or saline in small glass tubes which were kept in a thermostat. Water or saline was renewed daily and was aerated before use.

Weighings were carried out on a microbalance weighing to $\frac{1}{1000}$ mg. A technique similar to that described by Johnson (1937) was employed. Eggs were removed from the tubes with a pipette and placed in a watch-glass, and most of the water was pipetted off. Final drying was carried out with filter-paper until the surface of the eggs was dry. They would then roll freely in the watch-glass, when they were at once transferred to the weighing bottle and weighed. It was found convenient to use a small glass receptacle provided with a spout through which the eggs could be poured into the weighing bottle. The latter had a ground glass stopper, as trial weighings in which a loose stopper was used indicated that the balance was giving inconsistent readings because of water lost from the eggs by evaporation. Eggs were out of water for 4–5 min., and were out of the thermostat for about 30 min. each day; but allowance was made for this by keeping the tubes in a bowl of water at the approximate temperature of the thermostat while the weighings were being made in the weighing room.

INCREASE IN WEIGHT OF EGGS IN DISTILLED WATER

Nine batches of eggs were incubated at 20° C. in distilled water and weighed daily. The results are summarized in Table 1. The daily percentage increases in weight from weight at oviposition are calculated from the daily mean weights per egg, and are plotted in Fig. 1.

Table 1. *Increase in weight of eggs of* Corixa punctata *Illig. at 20° C. in distilled water*

Days after oviposition	No. of eggs	Total wt. nine batches (mg.)	Mean wt. per egg (mg.)	Minimum increase (%)	Maximum increase (%)	Mean increase and standard error (%)
0	99	22·036	0·222	0	0	0
1	99	22·236	0·225	0	2·2	0·9 ± 0·8
2	97	21·892	0·226	−0·1	3·3	1·6 ± 0·9
3	97	22·515	0·232	2·2	8·8	4·1 ± 1·9
4	97	25·892	0·267	13·7	25·5	19·9 ± 3·9
5	96	28·915	0·301	28·3	44·5	35·4 ± 5·5
6	96	30·457	0·317	34·8	52·6	41·9 ± 8·5
7	89	28·529	0·321	35·7	56·8	43·8 ± 6·9
8	88	28·537	0·324	37·6	58·7	46·1 ± 7·8
9	86	28·116	0·327	39·0	60·2	47·3 ± 7·8
10	80	25·967	0·325	36·5	58·7	46·6 ± 8·1
11	19	6·130	0·323	38·9	45·5	41·3 ± 2·7

The data show that under these conditions the eggs take 10–11 days to develop. For the first 3 days increase in weight is slight, between the 3rd and 6th days it is very pronounced, and thereafter it is again slow. The drop in weight on the 10th and 11th days is due to some eggs having hatched. Some of the eggs burst, possibly the result of rough handling, causing reductions in numbers. One batch on the 2nd day after oviposition showed an inexplicable decrease in weight which was confirmed by a second weighing. All batches were weighed on the 10th day but three were weighed early on the 11th. Hatching took place on the 11th day.

Similar experiments carried out in late June 1938 had to be discontinued as it was found that the eggs burst very easily and numbers in the batches were so depleted as to make further weighings inaccurate.

The data show a significant increase in weight each day, except between the 1st and 2nd, and 9th and 10th days. There was a significant decrease between the 10th and the 11th days, but this is probably due to the small number of eggs weighed on the 11th day.

Four batches of eggs were reared in distilled water at 14° C. The results are set out in Table 2 and the daily percentage increases in weight are plotted in Fig. 1. At this temperature the period of development was extended to 19 days; hatching commenced on the 20th day after oviposition. Increase in weight was most obvious between the 4th and 11th days, and the curve is not so steep as that for the eggs kept at 20° C. This fact may, of course, be due to there being only four batches of eggs as against nine in the first experiment; but it should be noted that the mean weight per batch at oviposition for the first experiment (at 20° C.) was 0·222 mg. as against 0·228 mg. for the second.

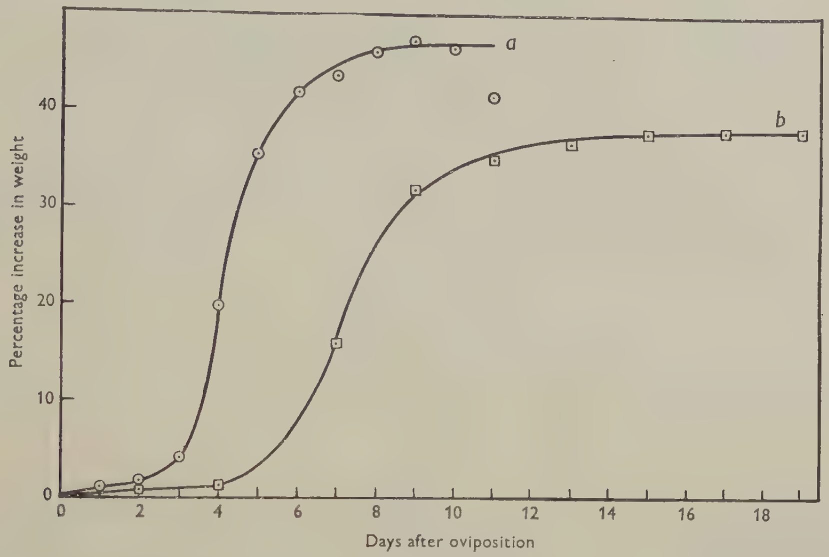

Fig. 1. Percentage increase in weight from weight at oviposition of eggs of *Corixa punctata* at 20° C. (*a*), and 14° C. (*b*), in distilled water.

There was a significant increase in weight between all weighings up to the 11th day, but there was no significant difference between weighings after the 13th day.

Table 2. *Increase in weight of eggs of* Corixa punctata *Illig. at* 14° *C. in distilled water*

Days after oviposition	No. of eggs	Total wt. four batches (mg.)	Mean wt. per egg (mg.)	Minimum increase (%)	Maximum increase (%)	Mean increase and standard error (%)
0	31	7·055	0·228	0	0	0
2	31	7·113	0·229	0·5	0·9	0·7 ± 0·1
4	31	7·111	0·229	0·8	1·8	1·2 ± 0·4
7	31	8·209	0·265	6·5	20·7	15·9 ± 5·8
9	31	9·316	0·300	22·8	37·3	31·7 ± 5·5
11	31	9·534	0·307	28·8	40·7	34·8 ± 4·2
13	31	9·653	0·311	31·2	42·4	36·6 ± 4·1
15	31	9·729	0·313	33·0	43·2	37·7 ± 3·5
17	31	9·748	0·314	33·5	43·2	37·8 ± 3·4
19	30	9·425	0·304	32·1	44·5	37·9 ± 4·5

THE EFFECT OF SALT WATER ON THE EGGS

Five batches of eggs were reared in solutions of sodium chloride in distilled water at 20° C. Four concentrations were used: 1·2, 0·8, 0·4 and 0·2% NaCl. Two batches were reared in the 0·2% solution and one batch in each of the others.

A preliminary experiment with a batch of 11 eggs in 2·0% NaCl solution showed that this salinity was too high. Within 24 hr. all the eggs showed obvious signs of plasmolysis, the contents being shrunken away from the chorions, and large vacuoles appeared near the apices of the eggs. Later, large dents appeared in the chorions and black patches showed on the surfaces. These changes were accompanied by losses in weight; the eggs showed a mean percentage decrease in weight on the 4th day of −4·3%. In the evening of the 5th day after oviposition, the eggs were placed in distilled water, and a weighing on the 6th day showed an increase in weight of +32·8%; but unfortunately the eggs had been killed, and weighings were discontinued after the 8th day, a maximum increase of 33·8% having been reached.

The results of the experiments at the other concentrations are given in Table 3, and the percentage increases are plotted in Fig. 2.

Table 3. *Effect of salt water on eggs of* Corixa punctata *Illig. at 20° C.*

Batch ...	*a*	*b*	*c*	*d*	*e*
Concentration of NaCl (%)	0·2	0·2	0·4	0·8	1·2
No. of eggs	8	6	8	7	6
Increase in wt. per egg from oviposition to final weighing (%)	53·1	43·8	27·4	15·8	4·3
Duration of incubation (days)	12	11	14	—	—
Total no. eggs hatched	8	5	7	Nil	Nil
Remarks	All hatched normally and successfully	1 burst two days after oviposition	1 failed to hatch; 2 had difficulty in hatching	1 partly hatched	None hatched

Absorption proceeded slowly for about 6 days in 1·2 NaCl and increases in weight were very small. None of the insects hatched.

In the 0·8% NaCl absorption was more rapid, but there was a marked falling-off in weight after the 6th day. Only one of the eggs showed signs of hatching on the 14th day after oviposition, but none hatched.

In the 0·4% NaCl absorption was even more rapid; seven of the eight eggs hatched (two having difficulty in hatching) and one failed to hatch.

In the 0·2% NaCl the two batches (six and eight eggs respectively) followed a course much closer to that of eggs in distilled water at the same temperature, maximum increases being between 45% and 55%. Eclosion occurred on the 11th and 12th days after oviposition. One egg in one batch burst 2 days after oviposition but all the others hatched successfully.

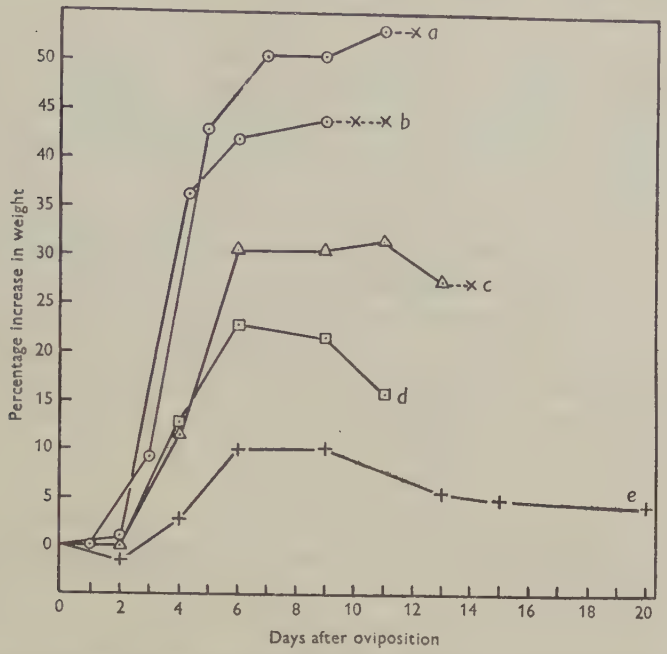

Fig. 2. Percentage increase in weight from weight at oviposition of eggs of *Corixa punctata* at 20° C. in solutions of NaCl: *a*, *b*, 0·2 %; *c*, 0·4 %; *d*, 0·8 %; *e*, 1·2 %. × indicates when hatching occurred.

DISCUSSION

Corixa punctata is not a normal inhabitant of saline or brackish waters, but on several occasions I have found it associated in small numbers with the typically euryhaline species *C. selecta* (Fieb.), *C. stagnalis* (Leach), *C. lateralis* (Leach) and *C. concinna* (Fieb.). It is unlikely, however, that *C. punctata* breeds in brackish water as it occurred only as the adult and its numbers were few. It is possible that the intolerance of the eggs to relatively high salinities might be a factor controlling the distribution of this species; but more work in this field is necessary.

Claus (1937) has studied the effect of salinity on the brackish water species *C. lugubris* Fieb. (= *C. stagnalis* (Leach)) and the two fresh-water forms *C. distincta* (Fieb.) and *C. fossarum* (Leach). Poisson (1924) describes his observations in field and laboratory on the tolerance to salinity of various aquatic Hemiptera. Some species are definitely euryhaline, and he gives a list of Corixidae which he has found in brackish or fresh water. *C. stagnalis*, *C. lateralis*, *C. striata* (Linn.), *C. venusta* (D. & S.) and *C. affinis* Leach, are most euryhaline, tolerating salinities up to 15–16 g. NaCl per litre; other species, including *C. punctata*, were found in waters containing 1–2, 1–3 and 2–4 g. NaCl per litre. He does not state, however, whether all these species were breeding under these conditions. From laboratory observations he concluded that salinity has no immediate harmful effect on the adults, but it does affect the eggs of certain species, e.g. *Notonecta* spp. For *N. viridis* Del. and *N. glauca* Linn. hatching is normal in 8 g. NaCl per litre; but for *N. obliqua* Gallen

(=*N. furcata* Fab.) and *N. maculata* Fab. hatching is difficult at this salinity; the larva sinks and cannot increase in volume as it does normally in fresh water. But for *Corixa stagnalis*, *C. lateralis*, *C. venusta* and *Naucoris maculatus* Fab. hatching is frequently normal in water containing up to 12 g. NaCl per litre. Poisson concluded that salinity is an important factor in controlling the distribution of many aquatic Hemiptera. Hutchinson (1930) makes similar observations, noting that *Corixa meridionalis* (Wall.) is able to exist in water containing up to 24·6 g. salt per litre.

Poisson has also investigated the effect of temperature on the embryonic period of development of some aquatic Hemiptera, including *C. lateralis*. At temperatures from 0 to 9° C. development is arrested, and the egg can be preserved for 10–12 months if development is not too far advanced. When returned to a normal temperature, he says, development of the egg continues. For this species the duration of embryonic development he gives as 14–16 days at 12–14° C. and 12–14 days at 16–20° C. My own results, given above for *C. punctata*, indicate 10–11 days at 20° C., and 19–20 days at 14° C.

The curves for *C. punctata* at 14 and 20° C. are very similar to those for *Popillia japonica* Newman (20 and 25° C.) (Ludwig, 1932) and for *Notostira erratica* L. (28° C.) (Johnson, 1937).

SUMMARY

The increases in weight of eggs of *Corixa punctata* Illig. in distilled water at two temperatures, 14 and 20° C., are described. Development is completed within 10–11 days from oviposition at 20° C., and within 19–20 days at 14° C.

The effects of various concentrations of sodium chloride on the eggs of this species are described. High salinities retard the absorption of water or may even destroy the eggs. The rate of absorption approaches more that of eggs in distilled water at the same temperature as the salinity is decreased. The eggs are able to tolerate salinities up to 0·4% NaCl.

The work of other authors on the effects of temperature and salinity on the eggs of aquatic Hemiptera is discussed.

This work was carried out in 1938–9 in the Department of Zoology, University College, Southampton. I am grateful to Dr C. G. Johnson for his advice and interest. I thank Prof. N. K. Adam, F.R.S. for extending to me the facilities of his Department, and Mr E. W. Balson for assistance with apparatus.

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INFLUENCE OF THE CORPUS ALLATUM ON THE OXYGEN CONSUMPTION OF ADULT *CALLIPHORA ERYTHROCEPHALA* MEIG.

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(*Received* 6 *December* 1948)

(With Six Text-figures)

I. THE PROBLEM

During the last ten years or so it has been demonstrated that in several insects the normal development of the ovaries in the adult is dependent on the presence of the corpus allatum (Wigglesworth, 1936; Weed (Pfeiffer), 1936; Pfeiffer, 1939; Thomsen, 1940, 1942; Vogt, 1943; Day, 1943; Joly, 1945, and Scharrer, 1946).

It was found that if the corpora allata are absent, the ovaries only develop to the stage at which the deposition of yolk begins. In a single case, *Dytiscus*, Joly (1945) showed that not only the deposition of yolk in the egg, but also the preceding growth of the ovarioles, is regulated by the corpus allatum. In all cases the influence of the corpus allatum on the ovaries was found to be hormonal.

Originally it was assumed that the hormone produced by the corpus allatum was a special gonadotropic hormone; some of the investigations, however, indicate that the corpus allatum hormone might have a more general effect on metabolism. This idea was first suggested by Pflugfelder (1937) and later on by Bodenstein (1943, 1944) and Day (1943). In 1945 Pfeiffer published a thorough investigation on the influence of the corpus allatum on the metabolism of the grasshopper *Melanoplus differentialis*. As Pfeiffer in her paper discusses this problem in detail, I need only refer to her publication. On the basis of quantitative analysis Pfeiffer arrived at the conclusion that the corpora allata regulates the development of the ovaries by means of a metabolic hormone. She does not preclude, however, that the corpora allata may secrete a specific hormone, which acts on the ovaries.

Some years ago I found that the corpus allatum in *Calliphora erythrocephala* Meig. influences the development of the ovaries and the size of the accessory glands in females and males (Thomsen, 1940, 1942). In accordance with the findings of Wigglesworth (1936) in *Rhodnius* and of Weed (Pfeiffer) (1936), and Pfeiffer (1939) in *Melanoplus* I assumed that the effect of the corpus allatum was due to the secretion of a gonadotropic hormone. The influence of the corpus allatum on the growth of the accessory glands of the female and male, together with the conspicuous accumulation of fat in allatectomized females suggested, however, that the corpus allatum might have a more general effect on metabolism as found by Pfeiffer in *Melanoplus*.

The problem is not so easily solved, for it is obvious that even if it were proved that the corpus allatum hormone has an effect on metabolism, it cannot be excluded that the corpus allatum may likewise produce a special hormone for the production of yolk in the egg.

Nevertheless, I found it of some interest to study the possible effect of the corpus allatum on metabolism, and for this purpose the oxygen consumption of normal and allatectomized females and males of *Calliphora* was measured.

II. TECHNIQUE

The oxygen consumption was measured by means of the Warburg apparatus (Warburg, 1926); 8% NaOH was used as the CO_2 absorbing agent. The whole apparatus was placed in a water-bath at a constant temperature of 25° C. The flies were put into flasks having a capacity of about 15–19 c.c., and before each experiment the apparatus was allowed to equilibrate for 20 min. with the taps open. During the equilibration and throughout the experiments the respirometers were rocked at a rate of 100 oscillations per minute. The water was stirred by means of two propellers. Each experiment lasted for 2 hr., and every 10 min. the manometers were read. (A stop-watch was used, and the manometers were read in the same sequence on each occasion.) If enough flies were available, eight flies were used in each experiment, and the flasks containing the operated individuals alternated with those containing the controls. In order to minimize the activity of the flies the blinds in the room were pulled down. The flies used for the experiments were of exactly the same age, i.e. 7 days, all the flies used in an experiment having emerged from the puparia within 2–3 hr. They were fed on meat, sugar and water and kept at 25° C. At an age of 7 days the females are as a rule mature, and the corpus allatum has grown to its full size (Thomsen, 1942, p. 336).

Before each experiment the flies were starved for 24 hr. in order that they might empty their intestines. Then the flies were weighed, the oxygen consumption being calculated on the live weight. After the experiments the flies were dissected and the intestine and crop examined for possible content of food. In all cases the intestine was found to be empty, although in several cases the crop contained some food; but as this applied to all the flies, including the controls, it was not considered of any importance.

The oxygen consumption of a total of 309 flies has been measured. Of these sixty-one flies were normal unoperated females and males, sixty-five were allatectomized females and males, sixty-five were operated controls to the allatectomized flies, fifty-eight were females and males with implanted corpora allata, and finally sixty were operated controls to these flies.

As the mortality of operated flies is rather heavy a great number had to be operated upon; so in order to obtain the above-mentioned numbers 147 flies were allatectomized, and 143 treated as operated controls, 110 flies each had three corpora allata implanted, and 106 were treated as operated controls, i.e. 506 have been operated upon in some way or other, and thus the mortality of the operated flies was about 50%.

Since the flasks containing the flies were submerged in the water-bath, and the blinds were pulled down, it was not possible to observe the flies during the experiments. As it was most likely that the resting periods corresponded to the lowest readings, the mean of the three lowest readings in each experiment was used for the calculations. In order to be reasonably sure of obtaining the lowest possible values, all experiments in which the difference between the three lowest values was greater than 0·2 (measured in mm. Brodie fluid) were excluded. Of the above-mentioned 309 experiments thirty-eight were discarded for this reason—i.e. 12%, ranging in the single series from 4 to 17%.

Usually, the values for the flies which were not at rest rose to 50, 100 or 200% of the values of resting flies. In a few cases the flies were so active that the values rose even higher, and the oxygen content of the flask was consumed long before the experiment was ended. (Naturally such experiments were excluded.) In a few cases a fly which gave a very high value was observed (by means of an electric torch), and it was always found that it was very active.

An example of the readings of one experiment is given in Table 1. As the flies were nearly always somewhat disturbed at the beginning of an experiment, the first reading was disregarded. In this case the readings are very constant, which indicates that the fly must have been at rest throughout the whole time of the experiment. As a rule the flies were surprisingly quiet.

Table 1

These figures represent the pressure differences (in mm. Brodie fluid) for periods of 10 min. in a single experiment lasting for 2 hr. with an allatectomized female.

(1, 2)	0·9	0·9	0·9	1·0	0·8	0·8	0·9	0·9	1·0	0·8	0·8

III. THE OXYGEN CONSUMPTION OF FEMALES

(1) *Normal* (*unoperated*) *adult females*

As a basis for the experiments on the possible influence of the corpus allatum on the oxygen consumption of *Calliphora* females, the oxygen consumption of thirty-one normal, unoperated, 7-day-old (25° C.) females was measured; five experiments have been excluded as they did not fulfil the claims mentioned above. The results expressed in oxygen uptake in cu.mm. O_2/100 mg. fly/10 min. are seen in Fig. 1. The oxygen consumption of these twenty-six normal unoperated females ranges from 21·2 to 38·6 cu.mm. O_2, the mean value being 28·3 ± 0·9.

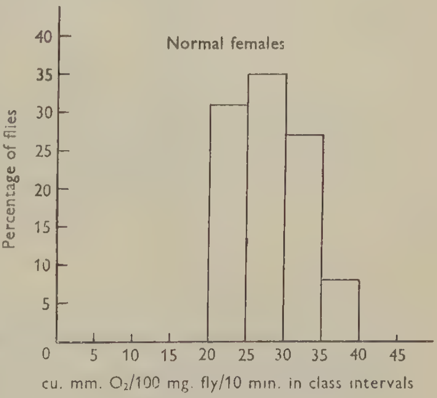

Fig. 1.

(2) *Allatectomized females and operated controls*

In this series the oxygen uptake of thirty-one allatectomized females was measured. The corpus allatum was removed when the flies were less than $7\frac{1}{2}$ hr. old (for the technique of allatectomy, see Thomsen, 1942, p. 346). In former experiments

(Thomsen, 1942, p. 347) it was found that even if the flies were allatectomized at that age, 14% of the flies developed their ovaries in spite of the removal of the corpus allatum. This fact was ascribed to the activity of the corpus allatum before the operation took place. In order to ascertain that the corpus allatum had not been active before the extirpation in the present experiments, the ovaries of the flies used for the experiments were examined after the experiment and the biggest egg-chambers measured. The length of the egg-chambers of these females only measured from 0·13 to 0·30 mm., with the exception of one fly whose egg-chambers measured 0·55 mm.; this case was excluded. The small size of the egg-chambers indicates that the corpus allatum had not been active before the removal.

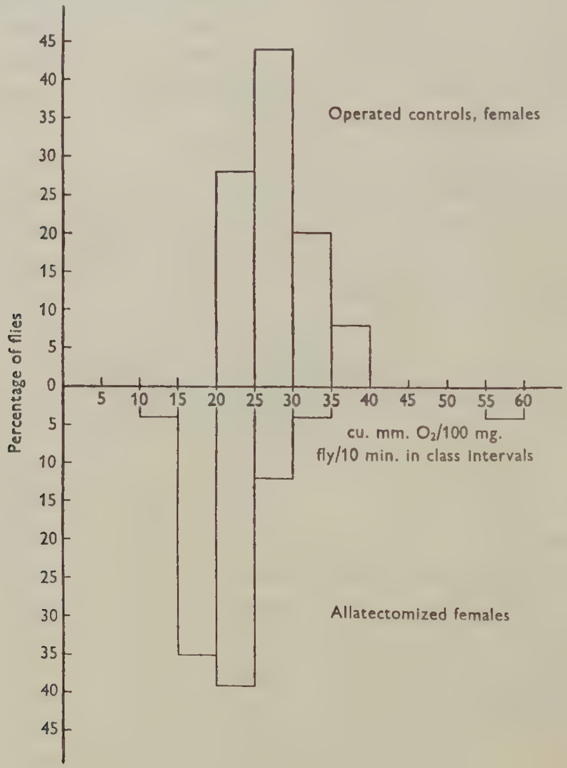

Fig. 2.

Five experiments, i.e. 16%, were excluded (p. 139). The oxygen consumption of the rest of the flies, twenty-six individuals, ranges from 13·1 to 56·6 cu.mm. O_2 (Fig. 2); 96% of the flies had an oxygen consumption which lay between 13·1 and 30·5 cu.mm. O_2, and only one fly had the abnormally high value of 56·6 cu.mm. O_2. As none of the normal, unoperated females had an oxygen consumption higher than 38·6 cu.mm. O_2, I think that this last value can safely be neglected in the calculation of the mean. As none of the normal unoperated females and males (p. 142) has an oxygen consumption which exceeds 40 cu.mm. O_2, I have disregarded all values above 40 in the calculation of the mean in all the various experiments. The mean of the oxygen consumption in the remaining twenty-five allatectomized females is then 21·3 ± 0·9.

As controls to the allatectomized females thirty females were operated upon in the same way as these, except that the corpus allatum was not removed, and their oxygen consumption was measured.

Five of these experiments were excluded. The remaining twenty-five flies had an oxygen consumption (Fig. 2) varying from 20·6 to 38·6 cu.mm. O_2, the mean value being 27·9 ± 0·9. As the mean value of the unoperated females was 28·3 ± 0·9, it is seen that the operation itself does not influence the oxygen consumption.

Comparing the mean value of the oxygen consumption of the allatectomized females with that of the operated controls, it is seen that the removal of the corpus allatum lowers the oxygen consumption by 24%, and as the difference was found to be statistically significant, it is obvious that the presence of the corpus allatum is necessary for the normal metabolism of the female Calliphora.

(3) *Females with three grafted corpora allata and operated controls*

Since removal of the corpus allatum decreased the oxygen consumption, it was to be expected that an implantation of corpora allata would result in an increase in the oxygen consumption, and such experiments were therefore carried out.

The flies used for the experiments were treated in the following manner: a number of flies, having emerged from the puparia within a few hours, were kept for 5 days at 25° C.; on the 5th day each of these flies had three corpora allata transplanted into the abdomen (for the technique see Thomsen, 1942, p. 357).* On the next day the food, except for water, was removed and on the 7th day the experiments were made. As the mortality is very heavy in flies operated upon twice, these flies retained their own corpus allatum. The flies which provided the corpora allata for the grafts were females from mass cultures in which oviposition had begun.

Twenty-seven experiments were performed and one of these was excluded. The oxygen consumption of these flies (Fig. 3) varied from 18·2 to 42·2 cu.mm. O_2. If the highest value is dis-

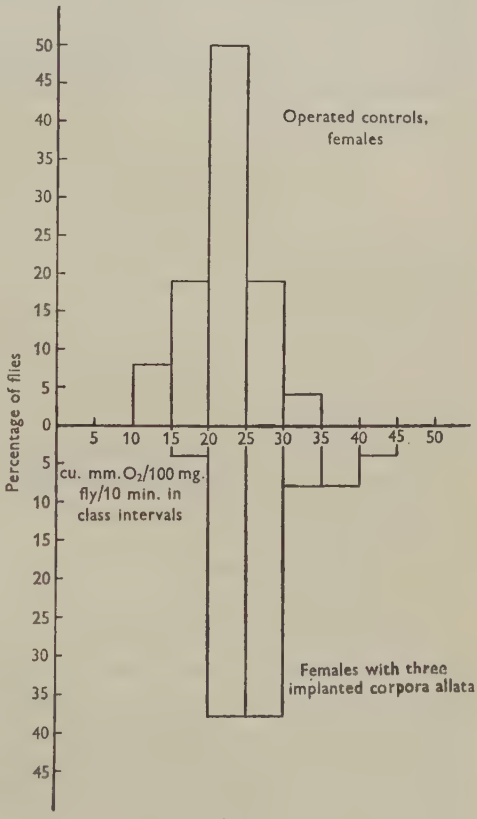

Fig. 3.

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* In the above-mentioned experiments the wounds in the abdomen were not sealed by means of rubber solution, but by means of paraffin, and care was taken that no spiracle was closed.

regarded (being higher than any value of normal unoperated females) the mean is found to be 26·3 ± 1·0.

As a control thirty-one experiments with females operated in the same way as those with implanted corpora allata were made. Of these experiments five, i.e. 16%, were excluded. The oxygen consumption of the remaining twenty-six flies varied from 11·2 to 31·2 cu.mm. O_2, the mean value being 22·1 ± 1·1.

Although these operated controls retained their corpus allatum, their oxygen consumption was 22% lower than the oxygen consumption of normal unoperated females. The decrease is certainly due to the operation, which is a rather severe one. The oxygen consumption of the females with implanted corpora allata was found to be 19% higher than the oxygen consumption of the operated controls. The difference between the means is 2·94 × the error on the difference.

As the difference was found to be statistically significant, these experiments seem to corroborate the experiments with allatectomized females.

The increase in the oxygen consumption due to the three implanted corpora allata is not as great as one would perhaps expect. It might be that the production of hormone from these implanted corpora allata is not as great as from corpora allata in their normal position. It might also be that the implantation, which was done by means of forceps, damages the corpus allatum to some extent.

Thus it is quite certain that the corpus allatum of adult female Calliphora *influences the metabolism.*

IV. THE OXYGEN CONSUMPTION OF MALES

(1) *Normal (unoperated) adult* Calliphora *males*

At first thirty experiments with normal unoperated males of *Calliphora* were undertaken; four of these experiments, i.e. 13%, were excluded. The oxygen consumption of the remaining twenty-six flies ranges from 19·8 to 37·4 cu.mm. O_2 (Fig. 4), the mean value being 29·0 ± 1·1.

It is interesting to note that the oxygen consumption of males is of the same order as the oxygen consumption of normal females.

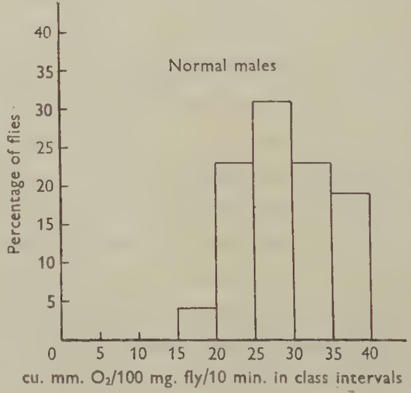

Fig. 4.

(2) *Allatectomized males and operated controls*

The experiments with allatectomized males of *Calliphora* and the operated controls are somewhat puzzling. The flies operated upon were less than 7 hr. old. Thirty-four experiments with allatectomized males were performed, but four of these, i.e. 12%, were excluded. The oxygen consumption of the remaining thirty flies (Fig. 5) varies from 15·9 to 63·6 cu.mm. O_2, twenty-six, i.e. 87%, of these values lie, however, between 15·9 and 35·8 cu.mm. O_2, whereas four values, i.e. 13%, were higher, being 42·5, 45·8, 49·6 and 63·6. As the highest value found in normal males was 37·4, these high results must be regarded as abnormal. The mean value of the experiments, is 28·0 ± 1·9, including the four abnormally high figures; if these are disregarded the mean becomes 24·6 ± 1·0.

The oxygen consumption of thirty-five operated controls was determined, but three experiments, i.e. 9%, had to be excluded, according to the principles enumerated (p. 139). The oxygen uptake of the remaining thirty-two controls ranges from 22·0 to 67·5 cu.mm. O_2. As it will be seen from Fig. 5, twenty-one of these males, i.e. 66%, had an oxygen consumption of the same order as normal unoperated males of the same age, whereas the oxygen consumption of eleven of the operated controls, i.e. 34%, was much higher. I have not been able to find the cause of this deviation from the normal. From a statistical point of view it is evident that these values cannot be disregarded, representing as they do 34% of the total amount of experiments. But, nevertheless, they are clearly abnormal.

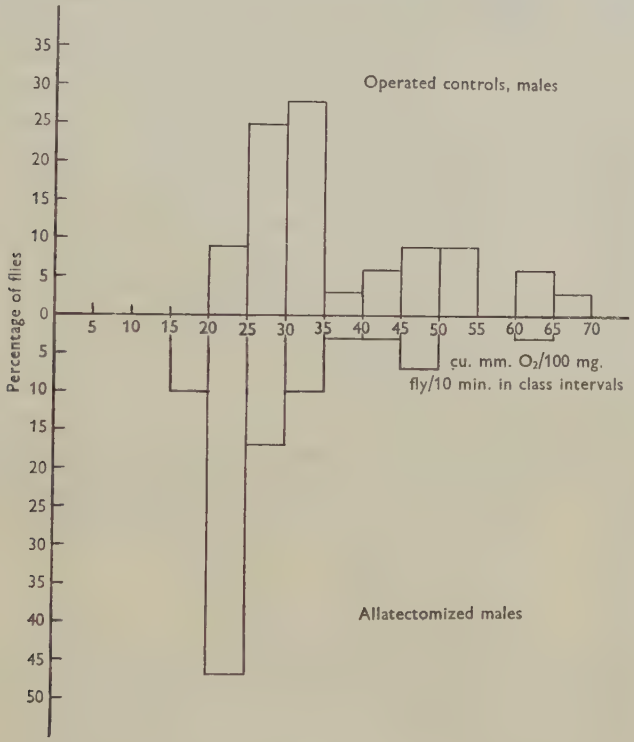

Fig. 5.

The mean value of all the thirty-two experiments was found to be 36·6 ± 2·3, a value which is 26% higher than that of normal unoperated males. As the flies were not anaesthetized, and as it was impossible to observe them during the experiments, the possibility that the flies in these last experiments have been constantly active cannot be entirely excluded.

The mean value of the twenty-one operated controls with the normal oxygen consumption was found to be 29·4 ± 0·9.

Disregarding the fact that the frequency curves show a great deal of skewness, the errors on the difference have been calculated by the ordinary method with the following results:

If all values of the allatectomized males and the operated controls are compared, it is found that removal of the corpus allatum lowers the oxygen consumption about 26%—if the values above 40 are excluded, the decrease in the oxygen consumption is only 16%. In both cases the difference was found to be statistically significant; in the first case the difference between the means is 2·88 times the error on the difference, in the second case 3·75 times.

As in the females, the hormone produced by the corpus allatum of adult males of Calliphora *was found to influence the normal metabolism.*

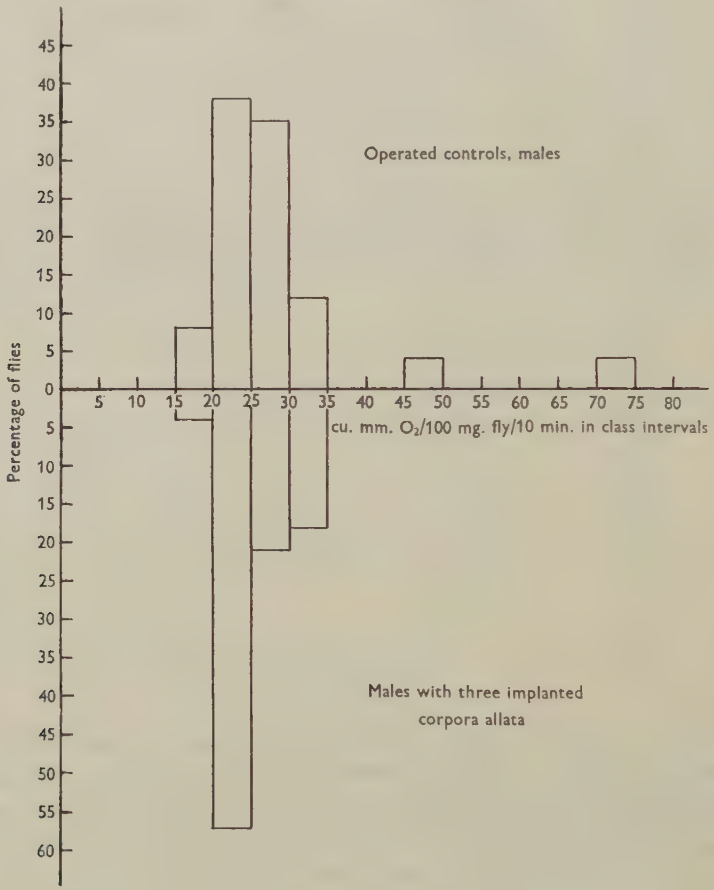

Fig. 6.

(3) *Males with three grafted corpora allata and operated controls*

Here again an attempt was made to increase the oxygen consumption by implantation of three corpora allata into males, which retained their own corpus allatum; the implanted corpora allata were provided from males of mass cultures older than 7 days. The males were treated in the same manner as the females with implanted corpora allata. Thirty-one experiments were performed, three of which, i.e. 10%, were excluded. The oxygen consumption of these males varies from 19·6 to 34·2 cu.mm. O_2 (Fig. 6), the mean value being 25·2 ± 0·8.

Twenty-nine experiments with operated controls were made; three of these, i.e. 10%, were excluded (p. 139). The oxygen consumption of the remaining twenty-six males lay between 15·0 and 72·2 cu.mm. O_2 (Fig. 6). Two values only were above the highest normal value (which I have arbitrarily chosen to be 40), measuring 49·5 and 72·2. If these two values are disregarded the mean value becomes 25·2 ± 0·8.

Just as was the case in females the operation itself was found to lower the oxygen consumption, but the decrease in these last experiments was only 13%—9% less than in the experiments with females. It is seen that the implantation of corpora allata did not increase the oxygen consumption of the males. Since the removal of the corpus allatum of males causes a definite decrease in the oxygen consumption, it is strange that the implantation of corpora allata did not increase the oxygen consumption to some degree. The result is disappointing and difficult to understand.

A possible explanation of this negative result may be that the corpora allata of older males are perhaps not as active as corpora allata from younger males. In previous experiments (Thomsen, 1942, p. 361) it was found that the corpora allata of males from mass cultures, in which oviposition had begun, caused egg-development in allatectomized females, and this was the reason why in these last experiments I used corpora allata from mass cultures. Afterwards I realized that I ought to have used corpora allata from 7-day-old males, especially because all the measurements of the oxygen consumption had been made on flies of that age. Since, however, the implantation of the tiny corpus allatum is rather difficult, and since the mortality is heavy, I refrained from making a new series of experiments.

V. DISCUSSION

I have found in the literature very few statements on the basal metabolism of adult flies. According to Davis & Fraenkel (1940, p. 406) the oxygen consumption of adult blow-flies (species?) which were *not* at rest was found to be 2–3 c.c./g. live weight per hour. The oxygen uptake of normal, unoperated, 7-day-old females and males of *C. erythrocephala* in my own experiments was found to be 1·7 c.c. O_2/g./hr. (females 28·3 cu.mm. O_2/100 mg. fly/10 min., and males 29·0 cu.mm. O_2/100 mg. fly/10 min.).

As it was not possible to observe the flies during the experiments, it cannot be stated with absolute certainty that they were at rest, but the great number of readings, totalling 3708, gave some practice in judging if the flies were quiet or moving about, and therefore I am almost certain that the low values which I found represent the basal metabolism of these flies. The value 1·7 c.c. O_2 in flies at rest is in good accordance with the oxygen uptake of flies which were not at rest, found by Davis & Fraenkel to be 2–3 c.c. (cf. my record on p. 139).

It is interesting to observe that the oxygen uptake of females and males of *Calliphora* was of the same order, since in *Drosophila melanogaster*, Kucera (1934) found that the oxygen consumption of females was higher than that of males. In this connexion it might also be mentioned that the oxygen consumption of the males of both the solitary and migratory phases of *Locusta migratoria* was higher than that of the females (Butler & Innes, 1936).

The experiments described above clearly show that the corpus allatum of *Calliphora*, both in the female and in the male, has an influence on the oxygen consumption. The decrease in the oxygen consumption of allatectomized females was found to be 24%. The decrease in the oxygen consumption of allatectomized males is more difficult to judge, since the values of the operated controls were much higher than those of normal unoperated males. If these abnormal values are disregarded, the decrease was found to be only 16%; if they are included the decrease is 26%. The fact that implantation of three corpora allata in females which retained their own corpus allatum only raised the oxygen consumption 19%, whereas the removal of one corpus allatum decreased the oxygen consumption 24%, is perhaps a little difficult to understand. But, as said above, it might be that the implanted corpora allata are not as active as corpora allata in their normal position.

It is interesting that the decrease in the oxygen consumption of allatectomized *Calliphora* is of about the same order as the lowering of the oxygen consumption of some vertebrates after thyreodectomy (Meyer & Ransom, 1945; Fleischmann, 1947; Hirsch, 1929).

In the females of *Calliphora* it was formerly found (Thomsen, 1940, 1942) that the deposition of yolk in the egg and the growth of the accessory glands were regulated by the corpus allatum. Whether the corpus allatum hormone which regulates the oxygen consumption is the same as that responsible for egg development, is still under dispute, but it is possible that the oxygen consumption and the growth of the accessory glands are influenced by the same hormone.

The theory that a special gonadotropic hormone is produced by the corpus allatum gains some support from the fact that a mutual influence of the corpus allatum and the ovaries exists; not only does the absence of the corpus allatum inhibit egg development, but castration results in a hypertrophy of the corpus allatum (Thomsen, 1940, 1942). Recently, Vogt (1947) has shown that the maturing ovaries of *Drosophila* produce a hormone which induces development of larval imaginal disks, and Vogt suggests that this hormone might be identical with the hypothetical hormone of the ovaries which restrains the growth of the corpus allatum.

In the male *Calliphora* the only function of the adult corpus allatum hitherto described (Thomsen, 1942) is the regulation of the growth of the accessory glands; no influence on the testes could be found. It might be supposed that the effects of the corpus allatum on the oxygen consumption and on the growth of the accessory glands are due to the same hormone.

Thus this investigation does not solve the problem of whether the corpus allatum of adult female *Calliphora* produces one hormone which is responsible for the deposition of yolk in the egg and another hormone which influences the metabolism, the only positive result being a definite proof that the corpus allatum has an influence on metabolism.

The decrease in the oxygen consumption may be due directly to the absence of the corpus allatum, but it might as well be due to the inhibited growth of the ovaries in the allatectomized females. If this were the case, the part played by the corpus allatum in the decrease of the oxygen consumption would be indirect. To test if the influence of the corpus allatum is a primary or a secondary one, the oxygen consumption of allatectomized and castrated females with implanted corpora allata should be measured. The mortality of such flies would be very heavy, owing to the severe operations, and since the operations *per se* would furthermore influence the metabolism to a high degree (p. 142), I have hesitated to make such experiments, although I realize that they might perhaps solve this problem.

In this connexion the experiments on males are of special interest. As mentioned above, it was found that the oxygen consumption of normal unoperated males was of the same order as that of normal unoperated females. This fact seems to show that the production of eggs, which in comparison to the production of spermatozoa needs much more material, is not necessarily accompanied by an increase in metabolism.

Furthermore in the males, in which no influence of the corpus allatum on the testes was found, the removal of the corpus allatum also caused a decrease in the oxygen consumption.

These two last mentioned facts suggest that the influence of the corpus allatum on the metabolism of females is a primary one.

In his investigation on the function of the ring-gland of adult *Lucilia sericata* Meig. and *Sarcophaga securifera* Villeneuve, Day (1943) found that the ring-gland has a profound influence on the fat-body, the oenocytes and the ovaries. On the basis of this result Day assumed that 'the effect of the ring-gland is probably primarily on some general metabolic process'. The ring-gland of the adult fly is, however, a complex organ comprising both the corpus cardiacum and the corpus allatum, as also pointed out by Day himself; and as Day in his experiments extirpated the whole ring-gland, some of the effects found might be due not to the corpus allatum, but to the corpus cardiacum. In my experiments on *Calliphora* only the function of the corpus allatum was studied, and thus it is rather difficult to compare my results with those of Day. It seems to me that Day does not always distinguish clearly between the function of the corpus allatum and that of the whole ring-gland.

The reported influence of the corpus allatum on the metabolism of *Calliphora* confirms the result found by Pfeiffer (1945) on *Melanoplus differentialis*. Her very thorough investigation based on observations of the size of the fat-body and the blood volume, and on quantitative determinations of the amounts of water, fatty acid content and residual dry matter, clearly showed that the corpus allatum of *Melanoplus* influences the metabolism. Pfeiffer draws the conclusion that the influence of the corpora allata on egg development may in part be accomplished by this metabolic hormone, and so she does not exclude the possibility that the corpora allata may in addition produce a special gonadotropic hormone.

Thus for the time being we can only say that in adult insects, at least in the grasshopper, *Melanoplus differentialis*, and in the blow-fly, *Calliphora erythrocephala*, the corpora allata regulate egg development *and* influence metabolism, but it is not possible to decide whether these two effects are brought about by the action of one hormone or of two different hormones.

SUMMARY

1. The possible influence of the corpus allatum on the metabolism of adult *Calliphora erythrocephala* was studied by measuring the oxygen consumption of allatectomized females and males by means of the Warburg apparatus.

2. As a basis for the experiments on operated flies, the oxygen consumption of normal unoperated females and males was measured. The oxygen consumption of the two sexes was found to be of the same order.

3. In the females allatectomy resulted in a decrease in the oxygen consumption of 24%.

4. Implantation of three corpora allata from females into females which retained their own corpus allatum increased the oxygen consumption by 19%.

5. In the males allatectomy likewise caused a reduction in the oxygen consumption, but the exact amount of the decrease was somewhat difficult to evaluate owing to a great number of abnormally high values in the operated controls.

6. Implantation of three corpora allata from males into males which retained their own corpus allatum did not raise the oxygen consumption. A possible cause of this negative result is discussed.

7. It was thus found that the corpus allatum is necessary for the normal metabolism of *Calliphora*.

I wish to express my sincere thanks to the chief of the Laboratory of Zoophysiology, Prof. Dr.phil. P. Brandt Rehberg, for his kind advice and for permitting me to use the Warburg apparatus in his laboratory. I would also like to offer my best thanks to Dr.phil. Hans Ussing for many valuable discussions. Likewise it is a pleasure to me to thank the technical assistants of the laboratory for their kind help. The Carlsberg Foundation has supported the work with a grant.

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THE UTILIZATION OF RESERVE SUBSTANCES IN *DROSOPHILA* DURING FLIGHT

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(*Received* 22 *December* 1948)

(With Four Text-figures)

The respiratory quotient in *Drosophila* during flight is about 1·0 (Chadwick, 1947), and the duration of flight to exhaustion varies with the age of the fly in the same manner as does the total glycogen—increasing steeply during the first 5 days of adult life, remaining at a maximum for about 10 days and then falling gradually until the fly is 5 or 6 weeks old (Williams, Barness & Sawyer, 1943). It appears certain that glycogen is the main or sole reserve substance consumed during flight.

It is the object of the present work to provide a histological picture of the distribution of the reserve substances and their mobilization during sustained flight. In the course of the work it has been possible to compare directly the efficiency of different substances as sources of energy for flight. *D. melanogaster* has been used throughout. The insects have been reared and maintained on standard maize-meal medium at 25° C.

DISTRIBUTION OF GLYCOGEN IN THE MATURE FLY

For the demonstration of glycogen the flies have been fixed in Carnoy's solution, transferred to absolute alcohol and split into two halves by a median longitudinal cut. The half insects have been stained in a saturated solution of light green in 90% alcohol and the glycogen revealed (i) by the iodine method as previously described (Wigglesworth, 1942), (ii) with Best's carmine. The two methods give identical results. Sections of Carnoy-fixed material have been cut in paraffin and celloidin and stained with Best's carmine.

Fig. 1 shows the general distribution of glycogen in the mature fly 1 week after emergence; and the details of its deposition in the cells.

(i) *Fat body.* The bulk of the glycogen is in the fat body of the abdomen. The fat-body cells have a few fat droplets around the nuclei, and occasional vacuoles with clear watery contents, but for the most part they are filled with huge peripheral deposits of glycogen (Fig. 1 J). Cells stained deeply with haematoxylin show the appearance seen in Fig. 1 H, with a cytoplasmic network ramifying through the glycogen deposits. Similar fat-body cells more or less laden with glycogen occur in the head, at the base of the legs, along the sides of the thorax, and in the scutellum.

(ii) *Halteres.* The next most conspicuous deposit of glycogen is in the large clear cells which fill the knob of the haltere (Fig. 1 D). These are almost certainly modified fat-body cells. They show ramifying strands of cytoplasm between the dense glycogen deposits like the fat-body cells but they contain almost no fat (Fig. 1 E).

(iii) *Flight muscles.* The third important location for glycogen is in the indirect flight muscles. This glycogen varies in amount but is usually less, or at least less conspicuous, than the massive deposits in the halteres. It occurs along the surface of the muscle masses, and around their points of insertion. Each muscle mass is made up of some ten to thirty lesser bundles each containing fifty to one hundred fibrils. Lesser deposits of glycogen occur between these smaller bundles. Finally, there are deposits of glycogen between the individual fibrils.

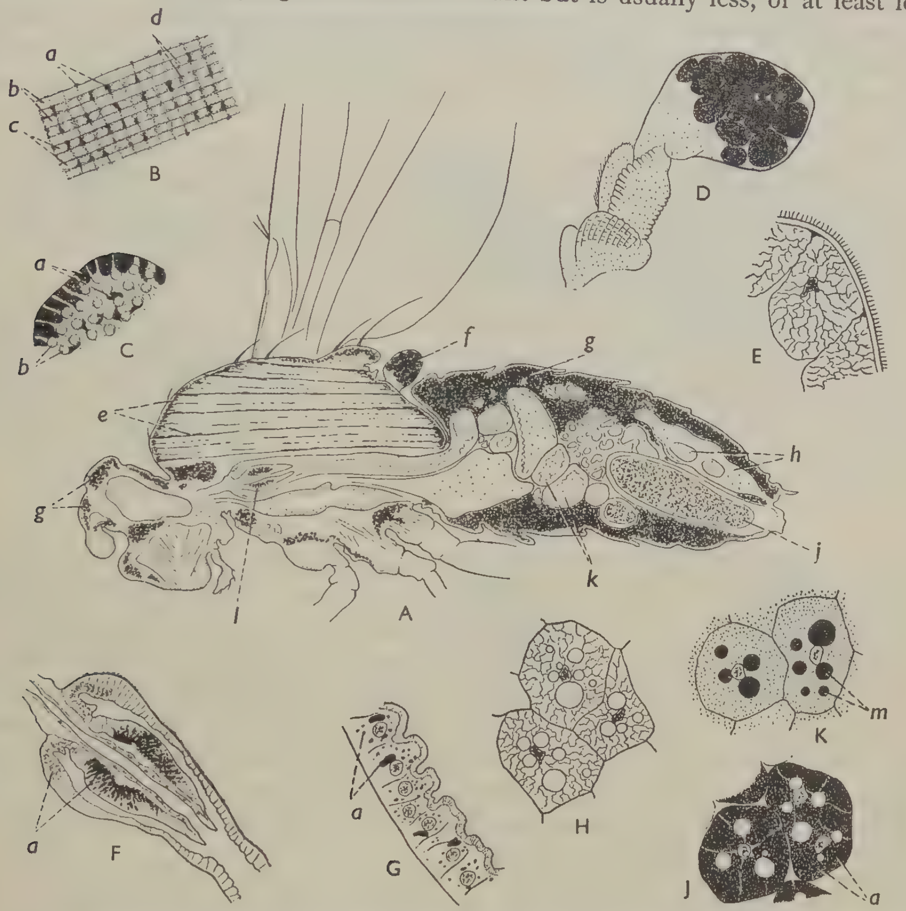

Fig. 1. Distribution of glycogen in the mature fly. A, sagittal section of female *Drosophila*, glycogen shown in black. B, longitudinal section of indirect flight muscle. C, transverse section through margin of fibril bundle in flight muscle. D, haltere. E, section through wall of haltere knob showing detail of the glycogen-containing cells. F, longitudinal section of proventriculus. G, section through wall of mid-gut. H, fat-body cells showing cytoplasmic network and fat vacuoles. J, fat-body cells showing glycogen. K, fat-body cells showing fat. *a*, glycogen; *b*, fibrils; *c*, sarcosomes; *d*, sarcoplasm; *e*, glycogen between muscle bundles and insertions; *f*, glycogen in haltere; *g*, fat body; *h*, rectum and rectal glands; *j*, oocyte with glycogen; *k*, mid-gut; *l*, proventriculus with glycogen; *m*, fat.

The structure and arrangement of these fibrils will form the subject of a separate paper; but it may be stated here that an oval sarcosome lies opposite each half of each sarcomere. Depending upon the method of staining, either the sarcosome or the intervening plasma may be caused to show up. In sections stained with Best's carmine and chlorazol black the sarcosomes are unstained, appearing as colourless vacuoles, and the glycogen is seen in the form of irregular deposits in the transverse meshwork of sarcoplasm which binds the fibrils together (Fig. 1 B, C). By the methods employed, no glycogen can be detected within the fibrils themselves, nor in the other muscles.

(iv) *Proventriculus.* Fine granules of glycogen can be seen in the cardiac cells of the mid-gut which secrete the chitinous peritrophic membrane (Wigglesworth, 1930; Siang-Hsu, 1947), but there is a far more conspicuous mass which fills the vacuolated cells of the oesophageal invagination—the plug against which the peritrophic membrane is moulded (Fig. 1 F).

(v) *Mid-gut cells.* Lastly there are comparatively small amounts of glycogen in the epithelial cells of the mid-gut (Fig. 1 G). None can be detected by this method in the rectal papillae, Malpighian tubes or oenocytes. There are traces only in the cells of the central nervous system. There are, of course, large amounts in the yolk of the developing eggs.

DISTRIBUTION OF FAT IN THE MATURE FLY

For the demonstration of fat the flies have been fixed in Bouin's aqueous mixture, cut in half as before, washed in 50% alcohol saturated with lithium carbonate and stained with B.Z.L. blue (Ciba). Sections have been prepared from flies fixed in Altmann's fixative and stained with Delafield's haematoxylin or with methylene blue.

Reserves of fat are far less conspicuous than those of glycogen. In the fat body there are clusters of comparatively small droplets around the nuclei (Fig. 1 K); there are small amounts in the epithelial cells of the mid-gut, and occasional very minute droplets occur in the cells of the haltere knobs. There is some diffuse staining of the flight muscles, notably in the sarcosomes, as well as in the other tissues, but no discrete droplets of stored fat.

CHANGES IN THE RESERVES OF GLYCOGEN, FAT AND PROTEIN IN THE YOUNG AND IN THE OLD FLY

In the newly emerged fly the 'larval fat body' is still evident and fills a great part of the abdomen. It is made up of spherical cells or groups of cells which are readily detached from one another (Fig. 2 A′). These cells contain large droplets of fat and refractile spheres of reserve protein.* Glycogen is scanty and takes the form of small peripheral deposits with granules scattered along the radiating strands of cytoplasm. The 'imaginal fat body' is made up of closely packed cells with small drops of fat and glycogen (Fig. 2 A). There is no demonstrable glycogen in the thoracic muscles or in the proventriculus, and there are traces only in the halteres (Fig. 2 D).

* The protein varies greatly in amount; the spheres are most numerous in the larger flies, irrespective of the sex.

During the next 48 hr., the larval fat-body cells diminish in size (Fig. 2B′); their protein droplets disappear, their fat is used up, and by 4 days after emergence no trace of these cells can be found. Meanwhile the adult fat body enlarges rapidly, particularly by the deposition of great quantities of glycogen (Fig. 2B). It reaches the state described in the mature fly in about 4 days (Fig. 2C). Likewise the glycogen in the halteres is gradually built up, reaching its peak at the same time (Fig. 2E, F).

In the fly at 4 or 5 weeks after emergence there is a general reduction in the amount of visible glycogen, but this amount still appears much greater than in the newly emerged fly.

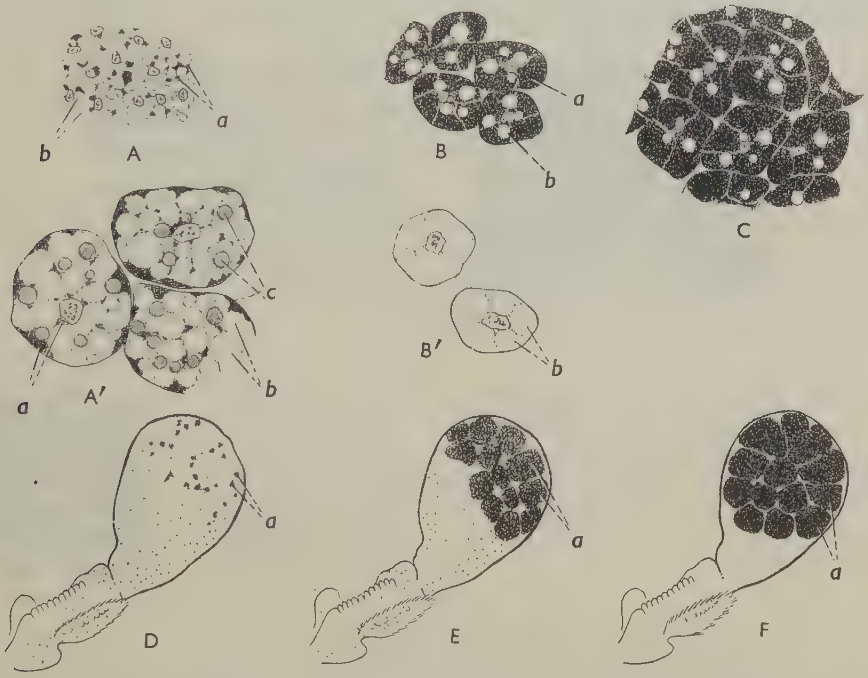

Fig. 2. Accumulation of glycogen in fat body and halteres in the young fly. A, imaginal fat body of newly emerged fly. A′, larval fat body of the same. B, imaginal fat body at 48 hr. B′, larval fat body of the same. C, imaginal fat body at 4 days. D, haltere of newly emerged fly. E, haltere at 48 hr. F, haltere at 4 days, showing progressive increase in glycogen. *a*, glycogen; *b*, fat; *c*, protein spheres.

UTILIZATION OF RESERVES DURING STARVATION

Mature flies, 1 week after emergence, have been given water alone and kept at 25° C. They survive for 2–3 days (cf. Hassett, 1948). By the end of 24 hr., glycogen is becoming markedly reduced in the fat body; reduction in the halteres is not so great, hence these deposits show up more conspicuously. Fat is reduced from the outset, concurrently with the glycogen.

In the later stages of starvation (2–3 days) glycogen disappears completely from the fat body (Fig. 3B), which is greatly reduced in size, and from the halteres and proventriculus. On the other hand, glycogen often persists in the thoracic muscles, which may contain plentiful deposits even at the time of death. In the moribund fly there are usually a few small scattered droplets of fat still remaining in the fat body (Fig. 3 B′). But it is clear that fat and glycogen are both consumed during starvation, at relative rates more or less proportional to the amounts present.

UTILIZATION OF RESERVES DURING FLIGHT

Flies of both sexes have been suspended by securing the dorsum of the thorax with wax to the head of a small entomological pin, using the technique of Hollick (1940). The pin is inserted into the cork of a 2 × 1 in. vial. The suspended insects will fly continuously for long periods. When they stop they are started again by smartly withdrawing the cork from the tube. Flight was maintained at a temperature of 20–22° C.

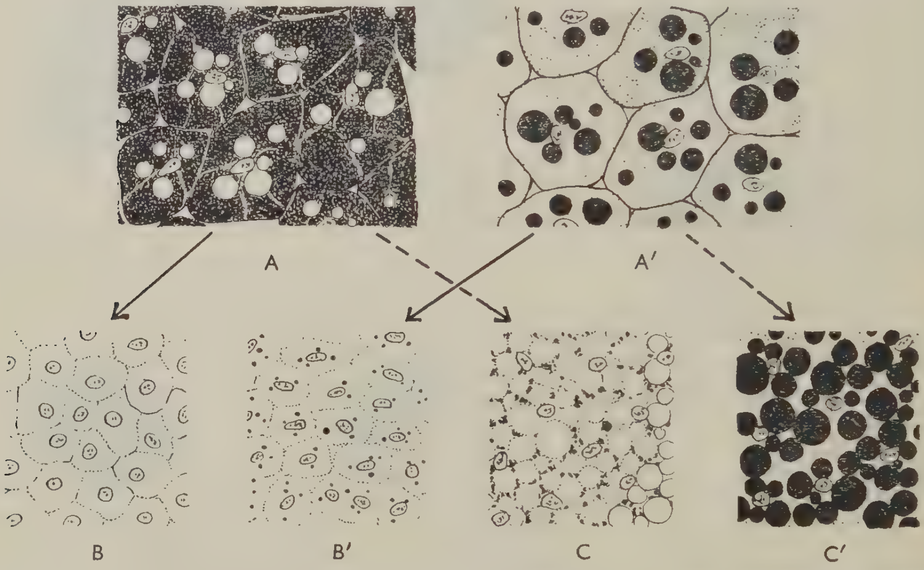

Fig. 3. A, fat-body cells of fly one week old showing glycogen. A′, the same showing fat droplets. B, the same after starvation until moribund; no glycogen remains. B′, the same showing minute fat droplets in the starved fly. C, fat-body cells in fly which had flown to exhaustion: granules of glycogen between the fat vacuoles. C′, the same showing fat droplets.

The duration of flight to the point of complete exhaustion varied widely within a group of flies from the same culture, but there was no obvious relation with sex. The age of the fly affected the duration of flight as already described by Williams *et al.* (1943); the period of flight is shorter in the very young and very old flies; but the total duration in *D. melanogaster* is much greater than that recorded by these authors in the larger species *D. funebris*. The results were as follows:

Flies 18–20 hr. old: 60, 75, 89, 92, 115, 127, 130, 145, 160, 170, 185, 187, 195: average 133 min.
Flies 1 week old: 195, 195, 200, 230, 245, 250, 275, 315, 320, 320, 325, 330, 345, 345: average 278 min.

Flies 4 weeks old: 50, 70, 75, 75, 80, 80, 80, 80, 85, 85, 95, 100, 105, 110, 115, 115, 120, 125, 130, 135, 180: average 100 min.

The corresponding values obtained by Williams *et al.* (1943) in *D. funebris* were 26, 110 and 20 min.

Flight behaviour of the exhausted flies. As they approach exhaustion the flies come to rest with increasing frequency, until they have to be restarted every minute or so. Then it can be noticed that instead of folding the wings the instant they cease to vibrate, the insect may stop with the wings extended as though it were still trying to fly. Finally, a point is reached at which, on withdrawing the cork, the wings are extended, make a few feeble beats and then stop. Exhaustion is then regarded as complete.

After a short rest, however, the exhausted insect may be induced to fly once more. It soon comes to rest, but will fly again for a shorter period still, usually only a few seconds, and the process can be repeated with gradually shortening periods of flight until exhaustion is once more complete. Until exhaustion is complete it is usually possible to restart flight within 1 sec. of the arrest. The duration of each short spell of flight can be timed, and the total duration of flight before exhaustion recorded. It then becomes evident that the total duration of flight of which the exhausted insect is capable is determined by the length of the preceding period of rest. The following is an example:

Duration of rest in min.	Successive flights in sec.	Total duration of flight in sec.
60	75, 4, 9, 5, 3	96
20	32, 10, 9, 5, 3, 3, 3	65
1	12	12
1	11	11
10	24, 5, 4, 3	36
20	33, 5, 3, 3	44
1	10, 3, 2	15
15	27	27

Naturally there is much individual variation. Fig. 4 summarizes the results obtained by these methods in a group of five flies of mixed sexes, some 1 week, some 4 weeks old. In spite of the variability in the results the increase in the duration of flight with the duration of rest is evident.

Changes in the glycogen reserves during flight. During the first hour of continuous flight there is a general consumption of glycogen in all the reserves. This almost disappears in visible form from the spaces between the muscle bundles. It is reduced in concentration throughout the vacuoles of the fat body—rather more strikingly in the thorax than in the abdomen. It is also reduced in the halteres. Everywhere the reduction is one of concentration in existing vacuoles rather than the complete discharge of certain deposits.

In flies which have reached the stage of exhaustion, or have made one or two post-exhaustion flights, there is a difference between old and young insects. In young flies, 5–7 days old, the exhaustion of glycogen in the fat body is much more complete; many of the cells are entirely devoid of glycogen, some have small deposits, and here and there are groups of cells with a considerable amount of glycogen still present (Fig. 3C). The halteres usually still show pale staining deposits. In old flies, 4 weeks old, there is much more glycogen remaining in the fat body; and the halteres, although their glycogen is being drawn upon, often still contain deeply staining deposits.

Evidently two factors are involved in the reduced duration of flight in the old insects: the total reserves of glycogen are smaller, and their mobilization is less efficient (cf. Williams *et al.* 1943). This is reflected in the more frequent stimuli needed to keep the older insects in flight, long before exhaustion is complete.

In both groups of flies the oöcytes in the female still contain large amounts of untouched glycogen. But besides this, the residual glycogen noted by Williams *et al.* (1943) in the exhausted flies comprises persistent reserves in fat body and halteres. 'Exhaustion' supervenes when the rate of consumption of glycogen during flight exceeds the rate of mobilization.

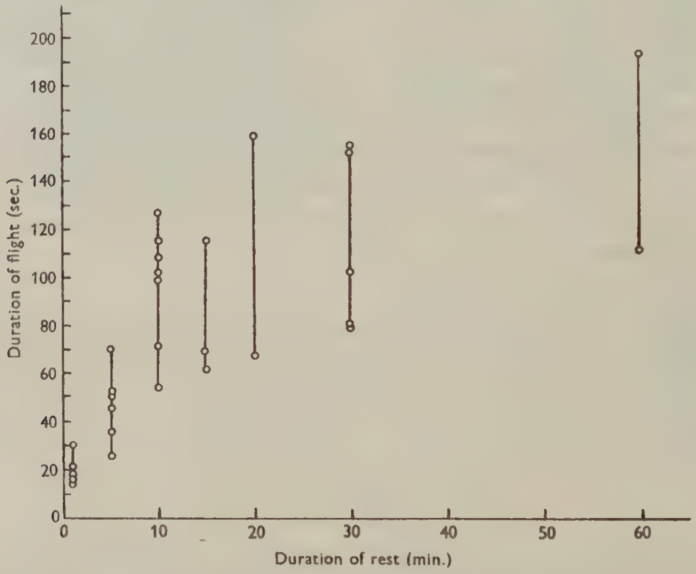

Fig. 4. Total duration of flight in exhausted *Drosophila* after varying periods of rest. Data from five flies of mixed age and sex.

Changes in the fat reserves during flight. In the normal insect, 5 days old, the droplets of fat in the fat body lie mostly around the nucleus at the centre of the cell with the distended glycogen vacuoles around (Fig. 3A, A′). In insects flown to exhaustion the cells are greatly shrunken, but the fat shows no apparent reduction—indeed, it often gives the impression of being increased because the fat droplets now fill the cell completely and in some places have run together to form larger droplets (Fig. 3C′).

Thus, whereas in starvation, glycogen and fat are consumed at parallel rates, during flight the glycogen is used up, leaving the cell laden with fat.

UTILIZATION OF SUBSTANCES FOR FLIGHT AFTER EXHAUSTION

It was suggested by Chadwick (1947) that the exhausted fly might prove good material for comparing the utilization of different substances as sources of energy for flight. The fly in this state usually refuses to drink plain water. If water is taken, it makes no difference to the capacity for flight. Desiccation is not the cause of exhaustion. If the exhausted insect is given a suitable sugar the ability to fly is restored in varying degree.

Method. The sugar is offered at the tip of a waxed capillary pipette of the type previously described (Wigglesworth, 1937). The concentration used, for all the substances tested, has been 10% w/v (that is, rather less than M/2 in the case of hexose monosaccharides). Substances which appear to taste sweet for the fly are taken up greedily as soon as they touch the anterior tarsi or the proboscis; about 0·1 mm.^{3} is absorbed within 30 sec. Other substances may require 1–2 min. for ingestion; and a few of the substances tried are not taken up unless 10% sorbose (which is not utilized by the fly, but apparently tastes sweet) is added to the mixture.

It has been found convenient to exhaust the insects on one day, to keep them overnight without feeding, to exhaust them again next morning and to record the total duration of flight following a rest of 1 min. They are then given the test substance, and any change in the flight behaviour, notably any change in the total duration of flight after a rest of 1 min., is observed.

Results. Before summarizing the results obtained with all the substances tested, five experiments will be recorded in full in order to illustrate the types of response.

Exp. 1 shows that glucose becomes available, in sufficient quantity to maintain uninterrupted flight, within 30–45 sec. after ingestion.

Exp. 1. Male fly 6 days old flown to exhaustion. Duration of flight after 1 min. rest: 7, 5, 3 (=15 sec.). Given just over 0·1 mm.^{3} of 10% glucose during 30 sec. immediately after flight. Flight was started at the end of this time and was then continuous for at least half an hour.

Exp. 2 shows that lactose has no effect on the duration of flight following a rest period of 1 min., at least up to 5½ hr. after ingestion; but at the end of this time continuous flight is restored within 60–90 sec. after the ingestion of maltose is begun.

Exp. 2. Female fly 6 days old flown to exhaustion. Duration of flight after 1 min. rest: 10, 3, 2, 2 (=17 sec.). 1 min. rest: 8, 3, 3 (=14 sec.).

Given 10% lactose; more than 0·1 mm.^{3} taken.

Immediately after feeding: 5, 4, 3, 2 (=14 sec.). 1 min. rest: 6, 4, 3 (=13 sec.). 5 min. rest: 19, 5, 4 (=28 sec.). 5 min. rest: 21, 4, 3 (=28 sec.). 30 min. rest: 34, 5, 3, 3, 2 (=47 sec.). 1 min. rest: 15, 4 (=19 sec.). 1 min. rest: 15, 4 (=19 sec.). 1 min. rest: 10, 3 (=13 sec.). 1 min. rest: 12, 4 (=16 sec.). 1 min. rest: 10, 3 (=13 sec.). 2 hr. 10 min. rest: 60, 5, 4, 3, 4, 3, 3, 2, 2 (=86 sec.). 1 min. rest: 11, 4 (=15 sec.). 1 min. rest: 9, 4, 2 (=15 sec.). 1 min. rest: 9, 3, 2 (=14 sec.). 2 hr. 15 min. rest: 45, 5, 4, 3 (=57 sec.). 1 min. rest: 9, 4, 3 (=16 sec.). 1 min. rest: 9, 3, 3 (=15 sec.).

Given 10% maltose; about 0·1 mm.^{3} taken in 30 sec.

Immediately after feeding: 11, 5, 6, 9, 9, 7; flight became continuous.

Exp. 3 shows that xylose will not support continuous flight; but after 2 hr. or less it is being assimilated and made available rapidly enough to support brief flights lasting 6–7 sec. and repeated indefinitely.

Exp. 3. Male fly 6 days old flown to exhaustion. Duration of flight after 1 min. rest: 7, 2 (=9 sec.). 1 min. rest: 7, 2, 2 (=11 sec.).

Given 10% xylose; about 0·15 mm.^{3} taken.

Immediately after feeding: 8, 3, 2 (=13 sec.). 1 min. rest: 7, 3, 2 (=12 sec.). 1 min. rest: 8 (=8 sec.). 1 min. rest: 10, 4 (=14 sec.). 1 min. rest: 9, 3 (=12 sec.). 1 min. rest: 7, 5 (=12 sec.). 15 min. rest: 28, 9, 5 (=42 sec.). 2 hr. 45 min. rest: 160, 10, 8, 5, 6, 6, 8, 7, 5, 6, 6, 7..., etc. 1 min. rest: 32, 7, 7, 6, 7, 7, 6, 6,... etc. 1 min rest: 11, 12, 10, 7, 7, 6, 7, 7, 6..., etc.

Exp. 4 shows that mannitol will not support continuous flight, nor maintain repeated short flights, but the total duration of flight after a rest period of 1 min. may be more than trebled.

Exp. 4. Female fly 1 week old flown to exhaustion.

Duration of flight after 1 min. rest: 12 (=12 sec.). 1 min. rest: 10 (=10 sec.). 1 min. rest: 9 (=9 sec.).

Given 10% mannitol; about 0·2 mm.^{3} absorbed during 2 min.

Immediately after feeding: 18 (=18 sec.). 1 min. rest: 18 (=18 sec.). 1 min. rest: 17 (=17 sec.). 1 min. rest: 14, 5 (=19 sec.). 1 min. rest: 17, 4 (=21 sec.). 1 min. rest: 15, 2 (=17 sec.). 1 min. rest: 32 (=32 sec.). 1 min. rest: 34 (=34 sec.). 1 min. rest: 25 (=25 sec.).

2 hr. later: made about 5 min. continuous flight. Then some long flights before exhaustion. 1 min. rest: 35, 11, 7, 3 (=56 sec.). 1 min. rest: 36, 3, 3 (=42 sec.). 1 min. rest: 32, 3, 2, 2 (=39 sec.).

Exp. 5 shows that glycine leads to a *shortening* of the duration of flight after a rest period of 1 min.

Exp. 5. Male fly 1 week old, flown to exhaustion.

Duration of flight after 1 min. rest: 6, 3, 2 (=11 sec.). 1 min. rest: 7, 3 (=10 sec.). 1 min. rest: 5, 3, 2 (=10 sec.).

Given 10% glycine; about 0·15 mm.^{3} taken in 45 sec.

Immediately after feeding: 8, 2 (=10 sec.). 1 min. rest: 5, 2 (=7 sec.). 1 min. rest: 4, 2 (=6 sec.). 1 min. rest: 3, 2 (=5 sec.). 1 min. rest: 2, 2 (=4 sec.). 1 min. rest: 3, 2 (=5 sec.). 1 min. rest: unable to fly in spite of making many apparent attempts. 1 min. rest: 2, 2 (=4 sec.). 1 min. rest: 4 (=4 sec.). 1 min. rest: 2 (=2 sec.). 1 min. rest: 2, 2 (=4 sec.).

Summary of results. All the substances studied fell within one or other of five categories. No doubt there are differences between the substances in a given category; but with a few exceptions (notably in the group giving continuous flight) they are obscured by individual variation among the insects. All substances were tested at the single concentration of 10%, from four to eight experiments being made with each. They are grouped as follows:

(i) Substances which will support continuous flight (Exps. 1 and 2): *glucose*, *fructose*, *mannose*, *sucrose*, *maltose*, *trehalose*.

Glucose is the most efficient; it will restore continuous flight within 30–45 sec. of ingestion. Fructose required 2–3 min. Mannose also required several minutes. Sucrose, maltose and trehalose required from 1 to $1\frac{1}{2}$ min.

(ii) Substances which will not support continuous flight but lead to repeated short flights of a few seconds which can apparently be continued indefinitely with a pause of a second or so between each (Exp. 3): *galactose*, *xylose*, *sorbitol*, *α-methyl-*D*-glucoside.*

(iii) Substances which lead only to an increase in the total duration of flight following a standard rest period of 1 min. (Exp. 4): *mannitol*, *glycerol*, *inositol.*

(iv) Substances which have no effect on the duration of flight (Exp. 2): *sorbose*, *rhamnose*, *arabinose*, *lactose*, *cellobiose*, *dulcitol*, *ethyl alcohol*,* *acetic acid** (as sodium salt), *lactic acid** (free and as sodium lactate), *β-hydroxybutyric acid** (as sodium salt).

(v) Substances which lead to a decrease in the total duration of flight following a standard rest period of 1 min. (Exp. 5): *glycine*, *alanine.*

THE RATE OF CONSUMPTION OF CARBOHYDRATE DURING FLIGHT

By feeding exhausted flies with a measured volume of glucose solution of known concentration, it has been possible to estimate directly the rate of consumption of carbohydrate during flight.

A fine wax-lined capillary pipette (Wigglesworth, 1937) has been used. It is graduated as follows. A column of mercury occupying about 5 mm. is introduced and its extremities marked. It is then discharged, mounted in Canada balsam, its diameter measured with a micrometer eyepiece and its volume calculated. A drop of water of this volume, that is, a drop which exactly fills the space between the two marks, is then introduced into the pipette and moved along so that the pipette is graduated into a series of segments of equal volume though of slightly varying length. Each segment is then bisected to give the unit actually employed—occupying a length of 2·5–3 mm. For measurement, each unit is divided into tenths by eye. In the pipette used, the unit had a volume of 0·024 mm.^3

Flies 24 hr. old were used. These were mounted and exhausted, kept overnight, and re-exhausted next morning so that even after a prolonged rest they were incapable of more than a few seconds flight. They were then given a measured volume of 5 or 20% glucose and kept flying continuously until completely exhausted again. In most cases the end-point was quite sharp, that is, within about 1 min. The results are summarized in Table 1.

The duration of flight for a given weight of glucose varies considerably, but it tends to be greater in the smaller insects. In the culture used, flies of mixed sex 1–2 days old had an average weight of 0·57 mg. As shown in Table 1 the mean duration of flight on 1 μg. of glucose is 6·3 min. (3·6–10·0).

It is interesting to compare this value with those deduced by Chadwick & Gilmour (1940) and by Williams *et al.* (1943). Chadwick & Gilmour used the much larger *D. repleta* (average weight 3·5 mg.). The average consumption of glycogen during a flight of 1 hr. (calculated from the oxygen uptake) is given as 0·1 mg. 1 μg. of carbohydrate will therefore last this larger fly 0·6 min. only.

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* Ingested by the fly only if 10% or so of sorbose was added.

Williams *et al.* used *D. funebris*. This had a glycogen content of 4·88 % of the live weight, which was reduced to 1·30 % of the live weight after 90 min. flight. If it be assumed that *D. melanogaster* (although a much smaller species) contains the same average percentage glycogen, and uses this at the same percentage rate, it can be calculated that it would consume 21·5 μg. in 90 min. So that 1 μg. would last 4·3 min.—a value which falls within the range observed above: 3·6–10·0 min.*

Finally, it is of some interest to form an estimate of the value of the glycogen contained in the knobs of the halteres as a reserve for flight. These knobs may be regarded as spheres about 0·11 mm. in diameter and with a volume therefore of 0·000695 mm.^{3} Assuming, as a very rough approximation, that the sphere has a specific gravity of 1 and may contain 50 % of glycogen, then the carbohydrate carried in the two halteres would amount to 1·4 μg. and would provide for about 4½ min. flight—say one-sixtieth of the total.*

Table 1

Insect used	Concentration of glucose (%)	Volume taken (mm.^{3})	Glucose taken (μg.)	Total duration of flight (min.)	Duration of flight in minutes per μg. glucose
A. Large male	20	0·204	41	152	3·6
B. Very small female	20	0·217	44	290	6·6
C. Average male	20	0·077	15	83	5·5
D. Rather small female	20	0·109	22	94	4·3
E. Average male	20	0·082	16	90	5·6
F. Small female	5	0·065	3	30	10·0
G. Average male	5	0·075	4	21	5·2
H. Small female	5	0·067	3	26	8·7
J. Large female	5	0·108	5	27	5·4
K. Average female	5	0·072	3·5	25	7·1
L. Average female	5	0·088	4	28	7·0

DISCUSSION

During starvation *Drosophila* utilizes both fats and carbohydrates. The fasting fly expends its energy in running around its container. There seems little doubt that fat and glycogen are both being used for muscular activity. It has long been known that grasshoppers lay down large stores of fat before making migratory flights; the fat content of the beet leafhopper (*Eutettix*) may be used as a measure of the distance over which it has flown (Fulton & Romney, 1940); and recently Krogh & Weis-Fogh (1948) have shown that the respiratory quotient of the desert locust *Schistocerca* during sustained flight equals 0·74–0·75. Yet the work of Chadwick & Gilmour (1940) and Chadwick (1947) has clearly proved, and the present observations show nothing to the contrary, that *Drosophila* during flight consumes only carbohydrate.

The difference is probably one of speed of metabolism. Glycogen, which is stored in large amounts in *Drosophila*, is the most readily available source of energy.

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* For the purpose of these approximations it has been assumed that glycogen has the same calorific value as glucose.

So long as it can be mobilized with sufficient rapidity to meet the high metabolic demands of the indirect muscles, flight is continuous. 'Exhaustion' supervenes when the reserve deposits are reduced to such a level that the rate of mobilization can no longer keep pace with the consumption. It is suggested that the conversion of fats into a form which will provide an immediate source of energy for muscular contraction in the fibrillar muscles cannot take place with the necessary speed and therefore the fly appears unable to use fats for flight.

The same argument may be applied to the substances given by mouth to the exhausted fly. It is highly probable that some of the substances in groups (iv) and (v), such as lactic acid, ethyl alcohol, glycine and alanine, can serve as sources of muscular energy. The two amino-acids are certainly efficient sources of glycogen in insects (Wigglesworth, 1942) as in other animals. The most likely explanation of their apparent failure to improve the powers of flight in the exhausted fly is that the chemical transformations necessary take too long a time.

In the case of substances in group (iii), like glycerol or mannitol, the speed of transformation is sufficient to increase the total duration of flight after a rest of 1 min., but not sufficient to support continuous flight. Substances of group (ii), like galactose or xylose, are transformed rapidly enough to give repeated brief flights but not quite rapidly enough to give uninterrupted flight. And among the group (i) substances it requires a little longer for fructose and mannose to become available in suitable form and adequate amount than it does for glucose. The delay amounting to half a minute or so, in the case of sucrose, maltose and trehalose is doubtless due to the time taken to effect hydrolysis, and the failure in the case of lactose and cellobiose results no doubt from the absence of the necessary enzymes.

The effect of glycine and alanine (group (v)) in actually shortening the duration of flight after a rest of 1 min. is interesting. Perhaps it signifies a diversion of carbohydrate, which would have been moved to the muscles, into some new channel. One thinks of the 'specific dynamic action' exerted by these amino-acids.

In general, the results agree very well with those obtained recently by Hassett (1948), who studied the effect of a long list of substances on the survival time of *D. melanogaster*. The numbers in brackets in the following lists represent the mean time of survival in days, as recorded by Hassett, in flies fed solely upon the substances used in the present study.

Group (i): glucose (16), fructose (18), mannose (14), sucrose (24), maltose (17), trehalose (21).

Group (ii): galactose (9), xylose (7), sorbitol (5), α-methyl-D-glucoside (6).

Group (iii): mannitol (6), glycerol (14), inositol (6).

Group (iv): water alone (2), sorbose (2), rhamnose (2), arabinose (2), lactose (2), cellobiose (2), dulcitol (2), ethyl alcohol (2), acetic acid (3), lactic acid (5).

Group (v): glycine (3), alanine (2).

In Hassett's experiments the presence or absence of digestive enzymes, the ease of metabolism, and the acceptability to the fly must all have been involved.

The results on *Drosophila* agree well also with those obtained in the mosquito larva *Aedes* (Wigglesworth, 1942), in which the efficiency of substances as sources of glycogen was compared. A curious exception is trehalose, which, oddly enough, was reported as being no better than galactose. Glycine and alanine were good glycogen formers.

The presence of dense deposits of glycogen, and the virtual absence of fat, in the cells of the haltere knob, is of little significance for the supply of energy. Perhaps the function of this glycogen is mechanical. Glycogen has a specific gravity of about 1·5. Its presence will increase the mass of the knob and add to its inertia. As a result it may improve slightly the efficiency of the haltere as a gyroscopic sense organ (Pringle, 1948).

SUMMARY

The chief reserve substance in *Drosophila* is glycogen. This forms dense deposits in the cells of the fat body and the haltere knobs. It is distributed throughout the indirect flight muscles as minute granules in the meshwork of sarcoplasm that fills the space between the fibrils and the sarcosomes. Somewhat larger masses lie along the surface of the fibre bundles and around their insertions. There is a large deposit in the proventriculus and small amounts in the cells of the mid-gut. The visible deposits of fat are much smaller and are confined to the fat body and the mid-gut cells.

During starvation, glycogen and fat are consumed concurrently. At the time of death (2–3 days) glycogen has disappeared completely, save in the thoracic muscles; only minute droplets of fat remain in the fat body.

In the insect which has flown to exhaustion (4–5 hr. in the mature fly) there is no apparent reduction in the stored fat. Glycogen is greatly reduced in all the deposits, but has disappeared completely only in the flight muscles and the proventriculus.

'Exhaustion' of the flying insect supervenes when the glycogen can no longer be mobilized rapidly enough to meet the metabolic demands of the flight muscles. Flight can be resumed for a brief period after the exhausted fly has rested; and the duration of flight increases with the duration of rest.

By observing the duration of flight after giving a measured quantity of sugar to the exhausted insect it is shown that 1 μg. of glucose will maintain *D. melanogaster* in flight for an average of 6·3 min.

The efficiency of substances as sources of energy for flight has been compared by giving them to the exhausted fly. Glucose will restore the capacity for continuous flight within 30–45 sec. of the commencement of feeding. Fructose, maltose, sucrose, etc., require a little longer. Galactose, xylose, etc., will allow repeated brief flights but will not support uninterrupted flight. Mannitol, glycerol, etc., merely increase the duration of flight after a standard period of rest. Lactose, sorbose, etc., have no effect. Glycine and alanine actually diminish the capacity for flight.

It is suggested that the apparent failure of fats, etc., to support flight in *Drosophila* is due to the comparatively slow rate of their metabolism.

It is suggested that the deposits of glycogen in the haltere knob may serve to increase the inertia of the haltere and so its efficiency as a gyroscopic sense organ.

I am indebted to Mrs A. Whittingham for technical assistance of many kinds and to Prof. A. Krogh and Dr T. Weis-Fogh for permission to quote their unpublished work.

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THE FERTILIZATION REACTION IN THE SEA-URCHIN EGG

A PROPAGATED RESPONSE TO SPERM ATTACHMENT

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(*Received* 21 *December* 1948)

(With Plates 4–6 and Three Text-figures)

INTRODUCTION

Two characteristics of the fertilization reaction in the sea-urchin egg, the block to polyspermy and the elevation of the fertilization membrane, necessitate the propagation of some disturbance round or through the egg from the point of attachment of the fertilizing spermatozoon. Changes in the appearance of the egg surface during the fertilization reaction have often been observed (see for example Just, 1919; Runnström, 1928; Ohman, 1945), but no objective records of these changes have been published. The nearest approach to a quantitative treatment is that of Moser (1939), who examined the dissolution of cortical granules following the fertilization of centrifuged sea-urchin eggs (*Arbacia punctulata*). The aim of the experiments described in this paper was to obtain objective and quantitative records of changes occurring at the surface of the egg after insemination. Two further series of experiments, which assisted in the interpretation of the main results, were carried out. First, the cortical response of the sea-urchin oocyte to insemination was examined. Secondly, an attempt was made to measure the rates at which sea-urchin spermatozoa move.

When considering whether some propagated change could or could not be the block to polyspermy, it is clearly necessary to know how often spermatozoa collide with an egg and what the probabilities are of such collisions being successful in the sense that fertilization follows. The frequency of collisions between an egg and spermatozoa can be calculated approximately if certain assumptions are made and provided that the density of the spermatozoa, their mean speed and the radius of the egg are known. The probability of a collision being successful can only be investigated by indirect methods. Though it is known that a spermatozoon may hit an egg, or even become stuck to its surface, without achieving fertilization, the only method of investigating this point appeared to be by taking advantage of the fact that oocytes, when inseminated, extrude a number of 'blebs' or papillae. These blebs may be caused by the lipolytic action of Androgamone III diffusing out of spermatozoa near the oocyte surface; or they may be an abortive response to sperm attachment, the oocyte being unable to propagate a block to polyspermy. Evidence will be adduced in favour of the latter hypothesis: the relationship between the number of blebs produced in a known time and the calculated number of collisions between an oocyte and spermatozoa in that time may give an indication of the probability that a successful sperm-egg collision will take place.

MATERIAL

Jelly-free eggs and spermatozoa of *Psammechinus miliaris*.

METHOD

Fertilization reaction. Dark ground cinematography.

Movements of spermatozoa. Dark ground cinematography. In this case a stop was inserted in the objective, reducing the numerical aperture to 0·1.

Artificial sea water, Ca *free.* 30 g. NaCl; 0·8 g. KCl; 6·6 g. $MgSO_4$; 0·495 g. $NaHCO_3$; H_2O to required salinity.

EXPERIMENTAL PROCEDURE

Cortical changes during the fertilization reaction. The egg was placed in a drop of sea water on a microscope slide with depressed centre; a cover-slip was then placed over the top of the drop. A micro-pipette containing a dilute sperm suspension was inserted under the cover-slip and a few spermatozoa placed near the egg surface. Photographs were then taken at 0·5 sec. intervals with an exposure of 0·25 sec. until the cortical change was completed or until the fertilization membrane was visible. Manipulations and sperm concentrations were arranged so that only a very small number of spermatozoa were placed near the egg.

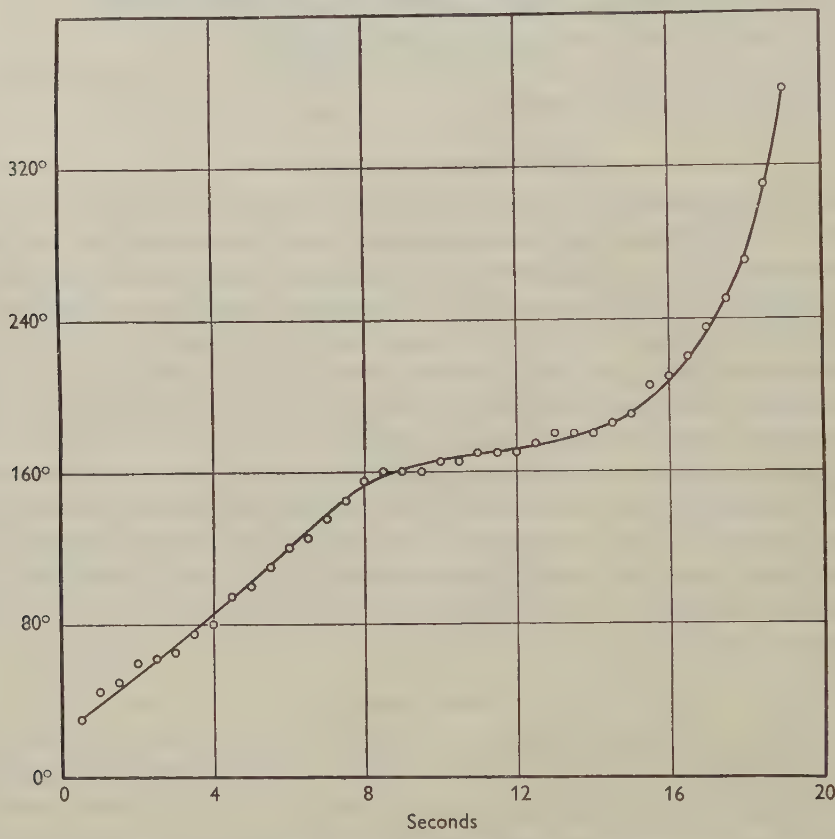
Movements of spermatozoa. A thin film of a dilute sperm suspension was spread on a slide. Photographs were taken at 0·5 sec. intervals, with an exposure of 0·25 sec.

Insemination of oocytes. Egg suspensions containing oocytes were inseminated in the usual way. After 5 min. the eggs were fixed in Bouin's fluid. Subsequently they were regressively stained in Ehrlich's haematoxylin, dehydrated, cleared in clove oil and mounted whole in xylol in balsam.

RESULTS

Cortical changes during the fertilization reaction. Pl. 4 is a series of photographs, at 0·5 sec. intervals, of an egg inseminated shortly before the first photograph. On frame 11 a small white arc can be seen at 5.30 o'clock, on the periphery of the egg. Between frames 11 and 49 the whole periphery of the egg becomes white. There are the following other points to note in this series of photographs: (1) A conical erection is visible on frame 24 at 5.30 o'clock. By frame 41 this structure has disappeared. (2) At the point on the egg surface where the structure described in (1) appears, and from this frame (24) onwards, there is a localized but transient decrease in the amount of light scattered at the periphery. The elevation of the fertilization membrane starts from this region. (3) There is a marked change in the shape of the egg during the reaction, indicating a change in the elastic or tensile properties of the cell surface. In particular the region round the point of sperm entry becomes flattened. (4) There is no significant change in volume. (5) There is a change in the brightness of the cytoplasm round the point of initiation of the reaction.

In Fig. 1 the development of the peripheral white colour is plotted against time. This cortical change is conveniently measured in terms of the angle subtended at the egg centre by the arc of the periphery which has changed colour. The speed at which the change is propagated is not uniform. From $t=0$ to $t=9$ (sec.) it is linear; between $t=9$ and $t=14$ there is a reduction in rate of propagation, while from $t=14$ to $t=19$, by which time the change has completely covered the egg surface, there is a marked increase in conduction rate. The egg whose properties during the fertilization reaction are recorded in Pl. 4 and Text-fig. 1 was selected because the conical erection referred to above is visible, which suggests that the spermatozoon entered the egg approximately in the plane of focus, that is at the equator, relative to the observer. In these conditions the rate at which the cortical change appears to be propagated is the true rate. If the spermatozoon enters the egg at a point which is not on the equator, the apparent and true conduction rates are different.



Text-fig. 1. Propagation rate of cortical change over egg surface. Ordinates, angle subtended at egg centre by arc of cortex which has changed colour; abscissae, time in seconds. T° C., 18.

As well as the egg illustrated in Pl. 4 and Text-fig. 1, twelve other eggs showing cortical changes were successfully photographed. The conduction times for their propagated cortical changes are given in Table 1. One egg (no. 4), as well as no. 3, exhibited the conical erection and was fertilized equatorially. In this case the cortical change was poorly defined, so that accurate measurement of the true conduction rate was impossible, though the increased velocity towards the end was obvious.

Table 1

Conduction times of cortical change
T° C., 18

Egg no.	Conduction time (sec.)	Remarks
1	17	—
2	15	—
3	19	Fertilization equatorial
4	32	Fertilization equatorial. Poorly defined change
5	12	—
6	33	—
7	19	—
8	13	—
9	16	—
10	19	—
11	19	—
12	17	—
13	15	—

The cortex of the egg is weakly birefringent, the birefringence being positive in the radial direction. The birefringence falls almost to zero when the propagated change has passed over the egg surface.

Movements of spermatozoa. Pl. 5 is a 0·25 sec. exposure of a dilute suspension of sea-urchin spermatozoa, using dark ground illumination. The directions of movement are random with an approximately Maxwellian speed distribution, the arithmetic mean speed being $190\,\mu$/sec. $\pm 7\,\mu$. Pl. 5 shows that the spermatozoa do not swim in straight lines but helicoidally. Only the projection of the movement of the sperm heads is seen in the photographs, the tails leaving no tracks. The frequency of the loops is about 40/sec. and it is reasonable to suppose that this is closely related to the frequency of the waves travelling down the tails of the spermatozoa. The hydrodynamic and energetic problems connected with this form of locomotion are not discussed in this paper.

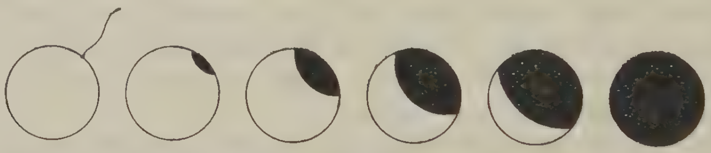
The figure $190\,\mu$/sec. refers to *translatory* sperm speeds, not total distances divided by time, which would give a considerably higher speed. In computing the translatory sperm speeds per second, the actual lengths of the tracks on the photographs have been multiplied by 4 and divided by the appropriate magnification figure. The fact that the tracks are the projections of motion in three dimensions has been ignored. The depth of focus is about $50\,\mu$, so the error involved is less than 6%. This method of measuring sperm velocities is not possible when the sperm suspensions have a normal density. The effect of dilution on the mechanical and metabolic activity of spermatozoa is well known (Gray, 1928; Emmens & Swyer, 1948; Rothschild, 1949). Consequently the recorded speeds may be somewhat low compared with normal speeds. At present no accurate method of measuring sperm speeds in dense suspensions is available.

Insemination of oocytes. Oocytes are usually found in egg suspensions of *P. miliaris* and the number of them can be increased by rough treatment of the ovaries. Suspensions were inseminated at sperm densities ranging from 10^5 to 10^7/ml. and fixed after 5 min. The reaction of these oocytes to insemination differs considerably from that of *Echinarachnius parma* described by Seifriz (1926). Pl. 6 (*a–d*) is a series of photographs, taken at 1 min. intervals, of an oocyte in a sperm suspension. The blebs or papillae which are protruded as a result of interaction between the oocyte and the spermatozoa have not been observed to retract or disappear as reported by Seifriz. Nor have large numbers of filamentous projections been noticed on the oocyte surface. In fixed and stained preparations spermatozoa can be seen in the blebs. A number of spermatozoa can also be seen on the surface of the oocyte, but not inside, in regions where no blebs have formed. Even when high concentrations of spermatozoa were used for insemination, the number of blebs formed was considerably less than the number of sperm-egg collisions. With a sperm density of 10^7/ml. there would be about 4500 sperm-egg collisions in 5 min.

If bleb-formation represents abortive activation, Ca-lack might be expected to inhibit the reaction, as it does fertilization. No blebs form in Ca-free sea water. For similar reasons acidification of sea water might inhibit bleb-formation. Oocytes inseminated in sea water at pH 5 show a significant reduction in bleb-formation, although less weight can be attached to this observation than that concerning Ca-free sea water because of the inhibitory effect on spermatozoa of acidified sea water.

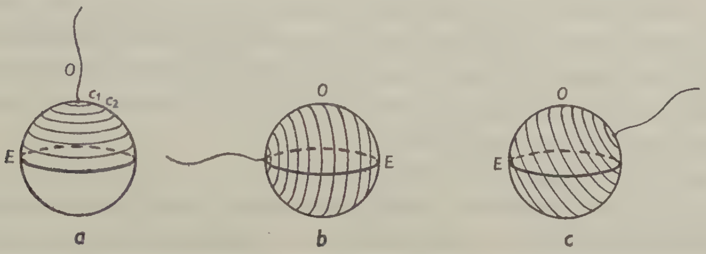
ANALYSIS

The kinetics of the propagated change. The observer, whose eye is directly above the north pole of the egg, sees the egg in optical section, the periphery of the section being the circumference of an equatorial great circle. When a spermatozoon fertilizes an egg, a change in surface structure spreads out in all directions over the surface of the egg, from the point of attachment of the spermatozoon (Text-fig. 2). The change may be likened to an expanding skin on the egg surface. If the spermatozoon fertilizes the egg exactly at the north pole, the periphery of the optical section of the egg will change colour instantaneously, because the surface change reaches all points on the equatorial great circle simultaneously. This is illustrated in Text-fig. 3*a* in which the periphery of the expanding skin is shown as a series of 'isochrones' depicting the position of the leading edge at various times after the fertilization reaction has started. If, however, the spermatozoon fertilizes the egg at the equator, the periphery of the optical section changes colour at the true rate at which the change is propagated. At points on the egg surface intermediate between the north



Text-fig. 2. Diagram to illustrate development of cortical change in an egg fertilized at 1.30 o'clock.

(or south) pole and the equator, the propagation rate, as judged by the time taken for the periphery of the optical section to change colour, will apppear to be higher than it actually is (Text-fig. 3*c*). Moreover, the form of the conduction curve will be altered. Even if the true conduction rate is uniform, this will not be so in the case of eggs which are not fertilized equatorially.



Text-fig. 3. Diagram showing passage of cortical change over egg surface. The observer at O sees the egg in optical section, the periphery of the section being the equatorial great circle E. *a.* Fertilization at the north pole. The whole of the cortical great circle E changes colour instantaneously. *b.* Fertilization at the equator. The cortical great circle E changes colour at the true rate at which the change is propagated. *c.* Fertilization at 2.0 o'clock. The change affects the cortical great circle E more quickly than the whole egg and the conduction rate apppears to the observer to be higher than it is. The concentric circles c_1, c_2, etc. represent the leading edge of the propagated change at times t_1, t_2, etc.

In a series of experiments, fertilization may occur anywhere on the egg surface though the probability is higher that fertilization will take place near the equator, relative to the observer above the north pole, than near the north or south pole. There will therefore be considerable variation in the time taken for the change to be propagated round the optical section of the egg, and in the form of the conduction curve.

Fortunately, it is possible to tell from the photographs, as in Pl. 4, if a spermatozoon fertilizes the egg at or near the plane of vision, because of the appearance of the small conical erection on the egg surface at the point where the cortical change starts. This is also the point at which the fertilizing spermatozoon becomes attached to the egg. This conical erection, which may or may not be identical with the fertilization cone of classical cytology, is not visible unless it is situated very near the equator. The true conduction curve can therefore be obtained by making measurements on eggs in which the conical erection is visible. In principle it might be possible to reconstruct the true conduction curve from data obtained when eggs are not fertilized equatorially. In practice, however, the reconstruction would be extremely difficult.

The conduction time in equatorially fertilized eggs should be longer than in all others, and with one exception this is the case (see Table 1). Variability in conduction times is the most probable explanation.

DISCUSSION

The nature of the cortical change. The cortex of the unfertilized egg of *Psammechinus miliaris* is hardly distinguishable from the cytoplasm. As the propagated change goes round the egg the cortex becomes white. This increase in cortical light scattering may be due to the formation of microscopic or submicroscopic granules. Moser (1939), however, states that cortical granules in the unfertilized egg of *Arbacia punctulata*, made visible by centrifugation, dissolve after adhesion of the fertilizing spermatozoon to the egg surface, the granules nearest the point of adhesion dissolving first. He also states that at 25° C. the process takes about 10 sec. to be completed, this figure having been obtained by calculating the arithmetical mean of a relatively small number of observations. No reference is made to the relationship between the appparent and true conduction rate. The largest value observed by Moser would be a more accurate estimate of conduction rate, but this is not given. The results described above are in contradiction with those of Moser, who found that fertilization reduced the brightness of the egg cortex.

Mechanism of the change. The change in cortical structure might be associated with the propagation of an action potential over the egg surface, or with the diffusion of some substance from the point of attachment of the fertilizing spermatozoon. The shape of the conduction curve does not support the action potential hypothesis. Furthermore, an action potential has never been observed in a spherical cell, even in ones which are the size of a pigeon's egg, and which therefore do not present great technical difficulties as regards measurement. Peterfi & Rothschild (1935) reported that after insemination of frogs' eggs, transient electrical changes were measured on the egg surface. Later, Rothschild (1946, p. 721) said that: 'Experiments of this type present such technical difficulties, however, that it is doubtful if much value can be attached to them until they are systematically repeated.' A more serious piece of evidence against the action potential hypothesis is that Rothschild (1938) found no potential difference across the surface of the sea-urchin egg before or after fertilization. It is difficult to see how an action potential, similar to those observed in nerve, muscle, or *Nitella*, could be propagated round the cortex of the egg, if there is no resting potential across the surface. As suggested by Rothschild, the apparent lack of potential across the surface of the sea-urchin egg may have been due to short-circuiting between the electrodes, the seal between the electrode inside the egg and the one in the sea water being inadequate. Although this might explain the lack of potential, it also follows that the propagation of the block to polyspermy is not dependent on the existence of a potential across the egg surface; because if the insertion of a micro-pipette into the egg could not be achieved without causing a serious electrical leak between cytoplasm and sea water, and if the fertilization reaction depends on the existence of the potential across the egg surface, fertilization should not occur after insertion of the pipette, though in fact it does. The alternative possibility, that the pipette never entered the egg, can, according to Rothschild, be rejected.

There is therefore no reliable experimental evidence that fertilization is associated with the propagation of an action potential over the egg surface. Nor is there any evidence of changes in cortical resistance after fertilization of sea-urchin or frogs' eggs (Cole, 1928; Cole & Guttman, 1942). Picken & Rothschild (1948) indirectly confirmed this observation for the frog's egg by measurement of their vapour pressure before and after fertilization. Cole (1938) found a 100% increase in cortical capacitance after fertilization of *Arbacia* and *Hipponoe esculentus* eggs. The marked changes in cortical structure which occur at fertilization make it hardly conceivable that there should be no changes in electrical properties at the same time, and a change in dielectric constant may perhaps predominate quantitatively over changes in resistance or potential. Even this concept has its objections, as Cole & Guttman (1942) found no change in the membrane capacitance of the frog's egg after fertilization.

As yet, there is not much evidence in support of the hypothesis that the change in cortical structure is caused by the diffusion of some substance from the point of attachment of the spermatozoon. But the time relationships of the conduction curve are of the right order for a diffusion effect and the actual shape of the curve is probably more consistent with a diffusion than an action potential mechanism.

The relationship between the observed change and the block to polyspermy. The block to polyspermy is usually assumed to be propagated over the egg surface at a much higher rate than the changes in cortical structure described in this paper. This assumption is based on arguments which do not rest on particularly firm foundations, i.e. that many spermatozoa collide with an egg in a short interval of time, for example, 1 sec.; and that the block to polyspermy must therefore travel over the egg surface, or through the egg, in a very much shorter time than 1 sec. Otherwise, the incidence of polyspermy would be much greater than it is. Gray (1931) suggested that the conduction rate might be comparable with that of an action potential in nerve or muscle. By analogy with smooth muscle he arrived at a conduction time of 10^{-5} sec. for the block to polyspermy in the sea-urchin egg. This argument raises three important questions: (1) How many spermatozoa collide with an egg in unit time? (2) Is every spermatozoon which collides with an egg potentially a fertilizing spermatozoon? (3) What is the incidence of di-, tri- and polyspermy, with varying densities of sperm?

The answer to question (3) is that no quantitative information is available, though Clark (1936) carried out some experiments on the induction of polyspermy in the eggs of various marine invertebrates. In her experiments the eggs were presumably surrounded by their jelly coverings. The actual sperm densities were not measured.

As regards the first question, the number N of spermatozoa that collide in unit time with an egg of radius a, immersed in a sperm suspension of density n, is approximately given by the equation

$$N = \pi a^2 n \bar{c},$$

where $\bar{c}$ = mean speed of the spermatozoa.

The derivation of this equation may be found in text books of physical chemistry. Its application to a suspension of spermatozoa involves a number of assumptions (see Appendix) which undoubtedly introduce errors. On the other hand, there does not seem to be any other way of approximating to a quantitative treatment of the problem. Substituting the observed mean speed 200×10^{-4} cm./sec. and $a = 50 \times 10^{-4}$ in this equation, $N = (1{\cdot}6 \times 10^{-6})n$. The value of n to be used must now be considered. A density of 10^9/ml. is a thick soup in which an egg is invisible. The same is roughly true of a suspension containing 10^8 sperm/ml. It may be assumed that densities ranging from 10^5 to 10^7 are normally used for insemination in the laboratory, while in nature, when males and females spawn simultaneously, the densities are probably of the same order. This means that for sperm densities of 10^5, 10^6 and 10^7/ml., the number of sperm-egg collisions per second will be 0·16, 1·6 and 16 respectively.

If the block to polyspermy is propagated round the cortex, or through the egg, at a high rate, there is no reason to suppose that a sperm-egg collision will be unsuccessful (question 2). If, however, the observed propagated change is the block to polyspermy, the probability of a collision being followed by fertilization must be low. In support of the latter view, many observers have noted that spermatozoa may collide with an egg without sticking to it, while on occasions spermatozoa stick to the egg surface without effecting fertilization. Moreover, considerations of specificity suggest that molecular groupings on the head of the spermatozoon and the egg surface must interlock in a particular way if fertilization is to occur. There are therefore some general grounds in support of the view that the probability of fertilization is lower than that of a collision.

The observed change takes about 20 sec. to travel over the egg surface, during which time there will be 3·2, 32 and 320 sperm-egg collisions at the three sperm densities. The spermatozoa which collide with the egg *after* the fertilization reaction has been initiated are not all potential producers of polyspermy, because about half of them will collide with those parts of the egg surface over which the propagated change has already passed (see Appendix). If it is assumed that a negligible percentage of polyspermy occurs at a sperm density of 10^7/ml., the conclusion is inevitable that either the observed change is not the block to polyspermy, or that only a fraction of the spermatozoa which hit the egg (of the order of 1/160th) are capable of fertilizing it.

The reaction of the oocyte to insemination. The oocytes were fixed 5 min. after insemination. If the cortex of the oocyte were unable to propagate a block to polyspermy, a number of spermatozoa should penetrate the surface in that time. This is in fact what happens, but the number which do penetrate is less than would be expected if each spermatozoon which collided with the oocyte during 5 min. were able to penetrate. Even when the sea water contained about 10^7 sperm/ml., in which case some 4500 spermatozoa should enter the oocyte in 5 min., the maximum number inside, or the maximum number of blebs produced, was less than 100.

In the Results section it was mentioned that each bleb contains a spermatozoon. The protrusion of these structures is clearly an abortive response by the oocyte to sperm attachment. If, as Seifriz (1926) suggested, the formation of these papillae were a reaction on the part of the oocyte before the spermatozoon had become attached to the surface, one would expect the number of processes to bear a close relationship to the number of sperm colliding with the egg. Examination of fixed specimens shows that this is not the case, as the surface of the oocyte often has a number of spermatozoa adhering loosely to it, though only a small fraction of these produced blebs. Once it has penetrated or has been engulfed, the spermatozoon may well be responsible for the 'softening-up' of the oocyte cortex through the lipolytic action of a substance diffusing out of the spermatozoon.

Why do so few spermatozoa enter the oocyte? The oocyte may propagate a block to polyspermy but at such a slow rate that some 50 or 100 spermatozoa have time to penetrate. But there is no evidence in support of this explanation, and it seems more likely that one of the ways in which the oocyte differs from the mature egg is in being unable to propagate any disturbance at all over its surface or through the cytoplasm. This applies not only to the block to polyspermy but also to the elevation of the fertilization membrane, since this also requires some propagated change in cortical structure. The reaction of the oocyte to insemination supports the possibility that only a small fraction of the spermatozoa which collide with the egg surface are able to initiate activation, or in the case of the oocyte, abortive activation.

The probability of an effective sperm-egg collision. If the probability of an effective sperm-egg collision is low, and evidence in support of this view has been suggested, two possible explanations of this fact may be put forward: 1. Spermatozoa may be 'muzzled' by combining with Gynogamone I in the sea water around the eggs, and so be unable to achieve fertilization or react with the cortex of the oocyte. 2. A particular orientation of the sperm head with respect to the egg surface may be necessary for the reaction to take place.

The first of these two possible causes is discussed by Rothschild (1947) and Tyler (1948). Although there is some doubt whether immature eggs produce those egg secretions responsible for the reaction between spermatozoa and iso-agglutinin, this has no bearing on the case where oocytes and ripe eggs are present together in sea water. The second explanation is bound up with the specificity of the fertilization reaction. This necessitates a complex of molecular or atomic configurations on the sperm head which makes it easy to appreciate the need for a particular orientation of the spermatozoon if the fertilization reaction is to take place. It may even be that only a small portion of the distal end of the sperm head has the required molecular configuration, in which case a collision between a spermatozoon and an egg may be unsuccessful unless this molecular pattern is geometrically able to fit into its appropriate opposite number on the egg surface.

CONCLUSION

Fol (1879) considered that the progressive elevation of the fertilization membrane—a propagated response to sperm attachment—was the block to polyspermy. This view is not accepted any more and in recent times the tendency has been to assume that the block to polyspermy is a rapid reaction, completed in a fraction of a second. The fastest propagated cortical change so far *observed*, however, is the one described in this paper, which takes about 20 sec. at 18° C. to pass over the egg surface. The facts are not entirely incompatible with the view that this cortical response constitutes the barrier to polyspermy, if we accept the hypothesis that only a fraction of the collisions between eggs and spermatozoa are followed by fertilization, either because of a previous reaction between the spermatozoa and egg secretions in the sea water, or because of the necessity for a particular orientation of the sperm relative to the egg surface if a successful interaction is to take place.

SUMMARY

1. After insemination of unfertilized eggs of *Psammechinus miliaris* and before the elevation of the fertilization membrane, a change in cortical structure is propagated over the egg surface or through the cytoplasm. When the fertilizing spermatozoon has become attached, the cortex progressively scatters more light when viewed under dark ground illumination. The cortex, which before fertilization is scarcely distinguishable from the cytoplasm, is white after the reaction is completed.

2. At 18° C. the change covers the egg surface in about 20 sec.

3. The conduction velocity is not uniform, there being a reduction in rate when the change is about halfway round the egg, after which there is a marked increase.

4. The cortex of the unfertilized egg shows a weak positive radial birefringence which disappears at the same time as the dark ground scattering appears.

5. Other changes in the egg structure occur at the same time, and are visible under dark ground illumination. These are: (*a*) a conical erection appears transiently at the site of sperm attachment; (*b*) the shape of the egg changes during the reaction; (*c*) there is a localized and transient decrease in light scattering at the point of entry of the spermatozoon, after the initial increase in cortical light scattering; (*d*) the fertilization membrane first appears at the point on the surface where the cortical change is initiated.

6. To investigate the possibility that the observed change might be the block to polyspermy, measurements were made of the speeds at which sea-urchin spermatozoa swim. A new technique has been evolved for this purpose. Spermatozoa were photographed under a special dark ground illumination system, with exposures of 0·25 sec. The heads of the spermatozoa trace out tracks on the film, the mean translatory speed being 190μ/sec. The motion is helicoidal, the frequency of oscillation of the illuminated sperm heads being about 40/sec.

7. By treating the sperm suspension as an assembly of gas molecules, a rough estimate has been made of the frequency of collisions between the spermatozoa and an egg. For sperm densities of 10^5, 10^6 and 10^7/ml., and for the observed translatory sperm speeds, the number of collisions between spermatozoa and those parts of the egg surface unaffected by the propagated change, after it has been initiated, is 1·6, 16 and 160 respectively.

8. Some preliminary experiments on the insemination of oocytes have been carried out to assess the probability of a sperm-egg collision being successful. The results were: (*a*) oocytes react to insemination by the extrusion of blebs or papillae, each of which is associated with a spermatozoon; (*b*) this reaction is inhibited by insemination in Ca-free sea water and partially inhibited by acidified sea water. It is concluded that the extrusion of papillae represents an abortive fertilization reaction, the oocyte cortex being unable to propagate a block to polyspermy. The number of papillae extruded is less than the number of sperm-oocyte collisions.

9. The experiments on the insemination of oocytes favour the possibility that attachment of the spermatozoon to an egg is not followed by fertilization unless

there exists a particular orientation, on a molecular scale, between the egg and sperm surfaces, and provided there has been no previous interaction between the spermatozoa and Gynogamone II. The low probability of fertilization that this implies is compatible with the hypothesis that the observed cortical change may be the block to polyspermy. Further experiments are needed to resolve this question.

APPENDIX

Mathematical considerations

(1) *The equation* $N=\pi a^2 n\bar{c}$. The equation is derived from the standard relationship $N=\frac{1}{4}ds\,dt\,n\bar{c}$, where N = number of collisions between particles at density n and with mean speed $\bar{c}$, and a surface ds, in time dt. The equation involves the following assumptions: (1) That a mean speed exists. It does. (2) That the movements of spermatozoa are random. This assumption is not justified in general as chemotaxis and the trap-action of the jelly round the egg (in nature) are not taken into consideration. Chemotaxis will tend to increase the number of collisions. The effect of the jelly is debatable. (3) That the sperm-velocity distribution function does not change during the period under examination. This assumption is probably justified in the presence of egg secretions. (4) That the three components of sperm velocity are independently distributed and have identical Gaussian distributions with means equal to zero. The assumption is unnecessary as the relationship can be shown to apply for any random distribution of sperm velocities.

(2) *The number of spermatozoa that collide with an unaffected part of the egg surface, after initiation of the propagated change* (*uniform conduction velocity*).

Let n = number of spermatozoa colliding with the egg per second.

Let T = total time taken for change to be propagated over the egg surface.

The number of spermatozoa colliding with the whole egg surface in δt sec. is $n\delta t$. The fraction of the egg surface covered by the change, after t sec., is

$$\frac{2\pi a^2(1-\cos t\pi/T)}{4\pi a^2} \tag{1}$$

$$=\tfrac{1}{2}(1-\cos t\pi/T), \tag{1.1}$$

where a = radius of sphere and $t\pi/T$ = half angle subtended at egg centre, by the 'polar cap', i.e. that part of the egg surface which has changed colour.

The uncovered fraction of the sphere surface is

$$1-\tfrac{1}{2}(1-\cos t\pi/T) \tag{2}$$

$$=\tfrac{1}{2}(1+\cos t\pi/T). \tag{2.1}$$

The number of spermatozoa colliding with unaffected parts of the sphere in time δt is

$$\tfrac{1}{2}n\delta t\,(1+\cos t\pi/T). \tag{3}$$

In time T, the number will be

$$\tfrac{1}{2}ndt\int_0^T(1+\cos t\pi/T) \tag{4}$$

$$=\tfrac{1}{2}nT. \tag{4.1}$$

Since during the time T, nT spermatozoa collide with the entire egg surface, half this number will collide with the unaffected part of the surface between the time that the change starts and when it completely covers the egg surface.

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EXPLANATION OF PLATES

PLATE 4

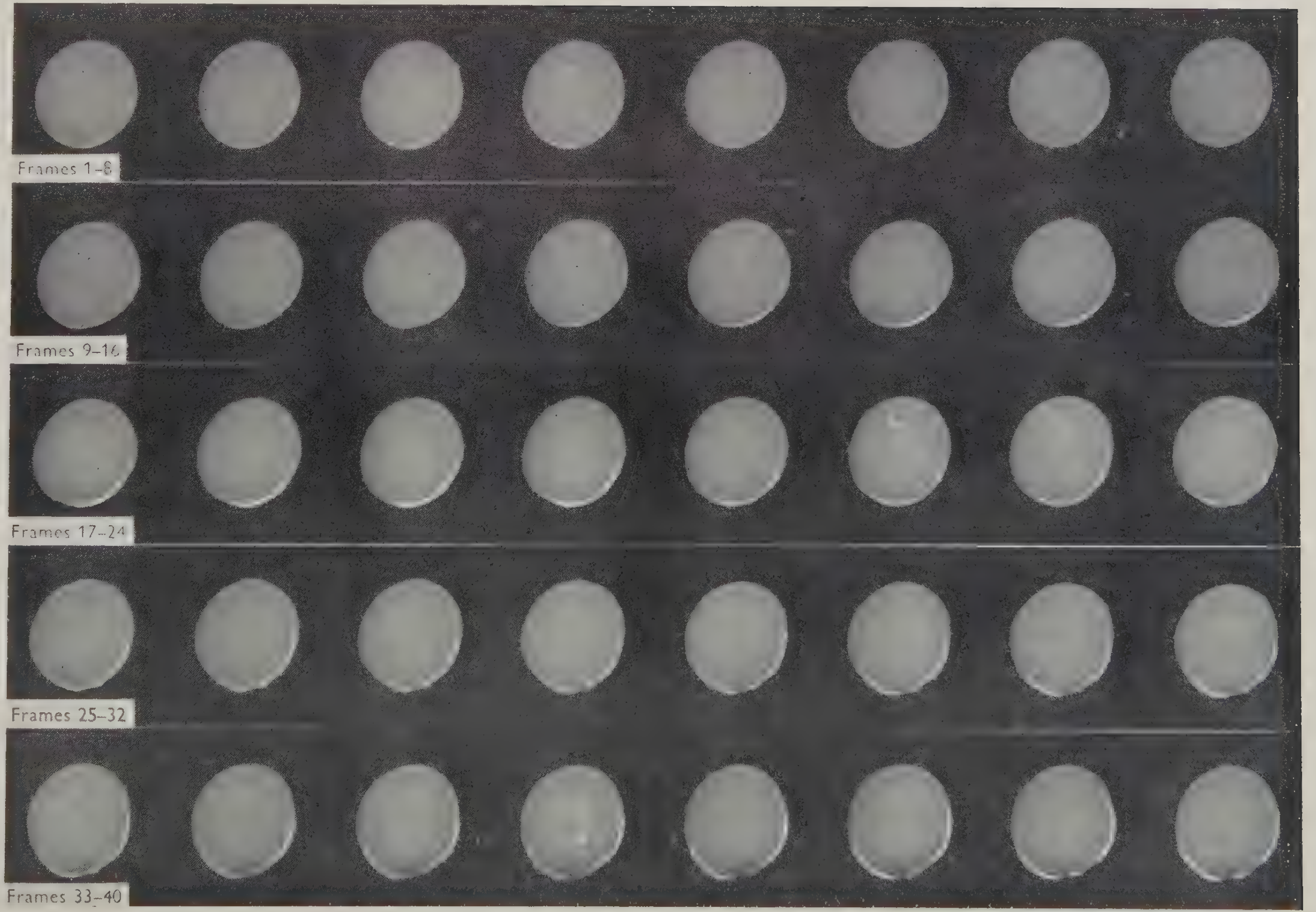
Propagated cortical change in egg of *P. miliaris* following insemination. Dark ground illumination. Exposure, 0·25 sec.; interval between photographs, 0·5 sec. Fertilization at 5.30 o'clock. Frame 11, beginning of cortical change; frame 24, appearance of conical erection; frame 55, first appearance of fertilization membrane (not visible in reproduction); frame 84, fertilization membrane completely but not yet symmetrically elevated. Diameter of egg, 100 μ. T° C., 18.

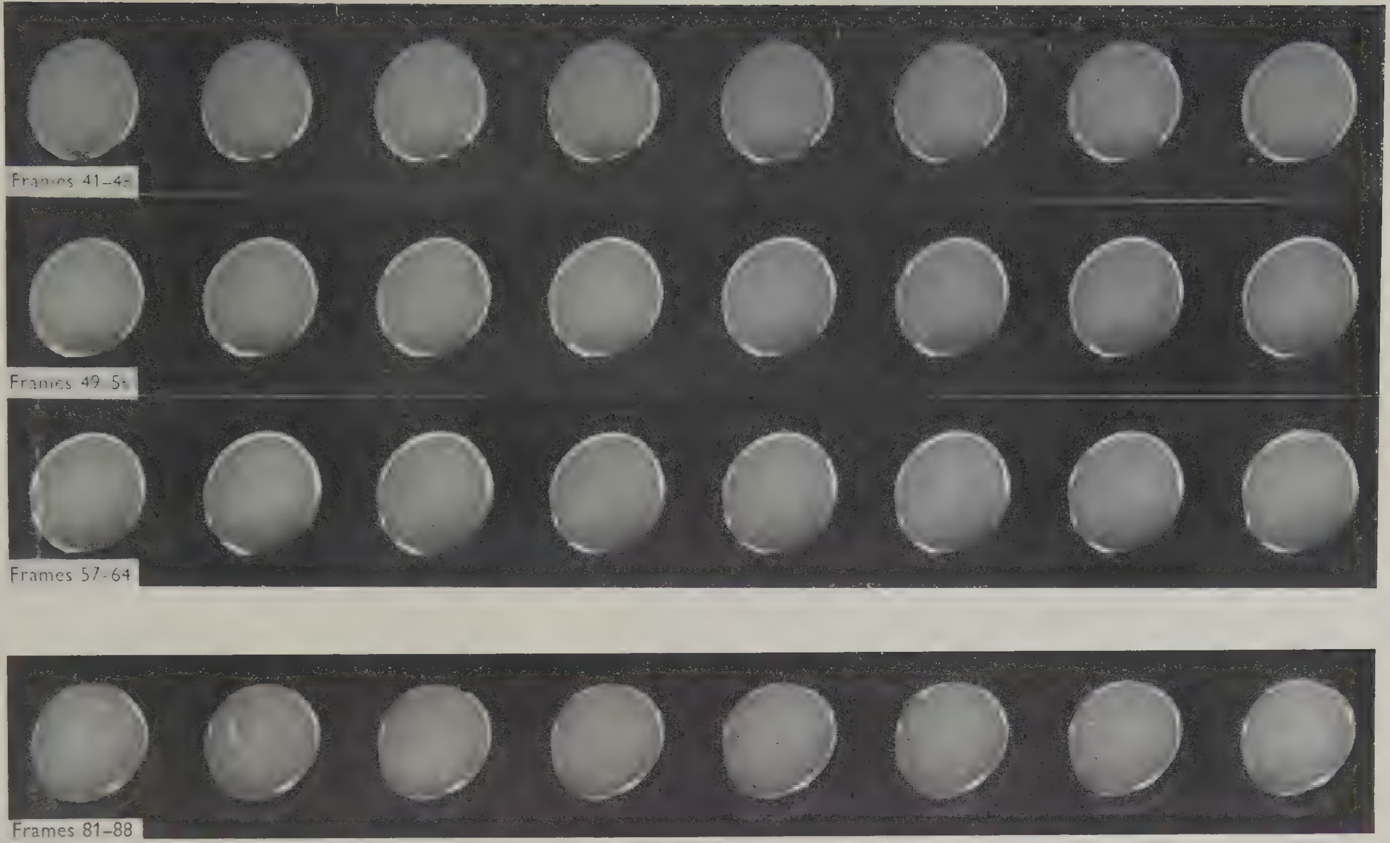
PLATE 5

Tracks of sperm heads under dark ground illumination. Exposure, 0·25 sec. Each track is approximately 50 μ long. T° C., 18.

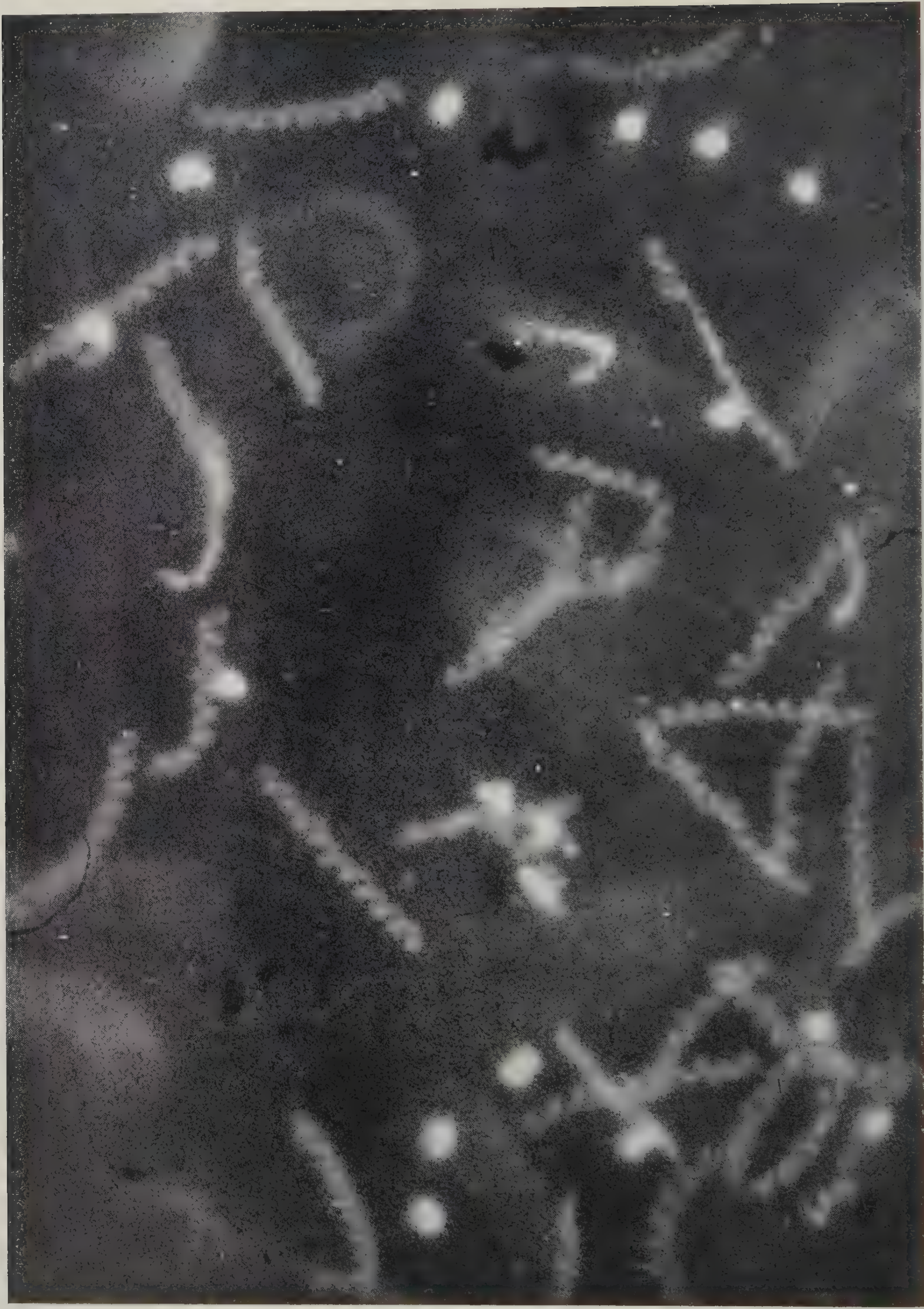
PLATE 6

Reaction of an oocyte to insemination; *a–d*, photographs, normal illumination, at 1 min. intervals. Diameter of oocyte, approximately 80 μ. T° C., 18.


Frames 1–8
Frames 9–16
Frames 17–24
Frames 25–32
Frames 33–40



ROTHSCHILD AND SWANN—THE FERTILIZATION REACTION IN THE SEA-URCHIN EGG



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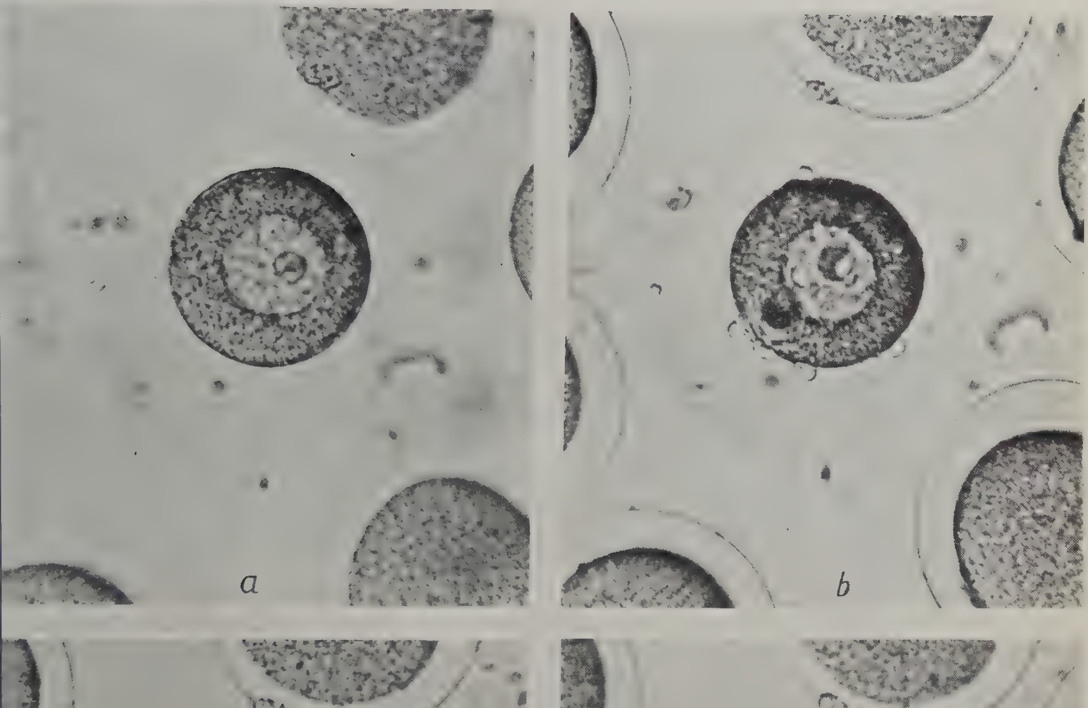

ROTHSCHILD AND SWANN—THE FERTILIZATION REACTION IN THE SEA-URCHIN EGG

THE FERTILIZATION REACTION IN THE SEA-URCHIN EGG

A NOTE ON DIFFUSION CONSIDERATIONS

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(*Received* 23 *March* 1949)

(With Two Text-figures)

The first sign that a sea-urchin egg has been fertilized is a change in cortical structure which spreads over the egg surface from the point of attachment of the fertilizing spermatozoon (Rothschild & Swann, 1949). The velocity of propagation is not uniform, there being a reduction in rate when the change approaches the equator of the egg relative to the point of attachment of the spermatozoon, and a marked increase when the cortical change has nearly covered the egg surface. The conduction mechanism of the cortical change might be similar to that occurring in stimulated nerve or muscle. Alternatively, it might be due to the diffusion of a substance, directly or indirectly derived from the head of the spermatozoon, round the cortex or through the cytoplasm, a change in cortical structure being effected by the arrival at the cortex of a particular concentration of the diffusing substance. If this hypothetical substance diffuses round the cortex and *not* through the cytoplasm, we must postulate a sympathetic reaction by 'affected' parts of the cortex, or an entirely separate cytoplasmic reaction, also initiated by the spermatozoon, to account for the intracellular changes that precede mitosis. By 'affected' parts of the cortex, reference is made to that fraction of the cortex which, owing to the arrival of a particular concentration of the diffusing substance, has changed its structure. On the other hand, if diffusion takes place *through* the cytoplasm, the one process might account for the two phenomena, cortical and intracellular changes, if cortex and cytoplasm are affected at different rates by the diffusing molecules.

Although the number of successful conduction velocity measurements was small, for reasons explained in the previous paper, it is of interest to examine the possibility that the cortical change is due to the diffusion of a substance, derived from the sperm head, round the cortex or through the cytoplasm. This possibility involves four questions:

(1) Can the diffusion hypothesis be rejected on the grounds that the experimental curve is quite inconsistent with it?

(2) If the answer to (1) is no,

- (*a*) Which fits the experimental data best, intracortical or intracellular diffusion? (There is evidence of a change in cytoplasmic structure during the propagated cortical change.)

(*b*) What concentration of the diffusing substance is needed at the cortex to effect the observed change in structure?

(*c*) What is the molecular weight of the diffusing molecule?

The equations appropriate to these conditions do not seem to have been solved, though a somewhat similar problem, involving radiation into the medium, has been solved by Carslaw & Jaeger (1947). As the detailed steps in the solutions are of no particular mathematical interest, they are omitted. The problems of diffusion through the cytoplasm or round the cortex, from a small source of a diffusing substance at the surface, are conveniently formulated in terms of heat conduction.

I. DIFFUSION THROUGH THE CYTOPLASM

An instantaneous point source of heat is placed on the inner surface of an adiabatically insulated sphere containing a homogeneous isotropic conducting medium with temperature initially zero everywhere. What is the subsequent flow of heat?

$$\frac{\partial V}{\partial t} = \kappa\left\{\frac{\partial^2 v}{\partial r^2} + \frac{2}{r}\frac{\partial v}{\partial r} + \frac{1}{r^2}\frac{\partial^2 v}{\partial \theta^2} + \cot\theta\,\frac{\partial v}{\partial \theta}\right\} \quad (0 < r < a). \tag{1}$$

Boundary condition:

$$\left(\frac{\partial v}{\partial r}\right)_{r=a} = 0.$$

Solution:

$$(V)_{r=a} = V_\infty + \tfrac{2}{3}V_\infty \sum_{n=0}^{\infty}\sum_{\alpha} \frac{2n+1}{1 + \dfrac{1}{4a^2\alpha^2} - \dfrac{(n+\frac{1}{2})^2}{a^2\alpha^2}} P_n(\mu)\, e^{-\kappa\alpha^2 t}, \tag{2}$$

a = radius of sphere,

V_∞ = temperature at $t = \infty$,

$\mu = \cos\theta$,

κ = diffusion constant,

α = positive non-zero roots of the equation $\alpha a J'_{n+\frac{1}{2}}(\alpha a) = \frac{1}{2} J_{n+\frac{1}{2}}(\alpha a)$.

II. DIFFUSION IN THE CORTEX

An adiabatically insulated spherical shell, external and internal radii a and b respectively ($0 < b < a$), *with initial temperature everywhere zero, is heated by placing an instantaneous point source of heat on the outer surface of the shell. The shell contains a homogeneous isotropic medium. What is the subsequent flow of heat?*

$$\frac{\partial v}{\partial t} = \kappa\left\{\frac{\partial^2 v}{\partial r^2} + \frac{2}{r}\frac{\partial v}{\partial r} + \frac{1}{r^2}\frac{\partial^2 v}{\partial \theta^2} + \cot\theta\,\frac{\partial v}{\partial \theta}\right\} \quad (a < r < b). \tag{3}$$

Boundary conditions:

$$\left(\frac{\partial v}{\partial r}\right)_{r=a} = 0, \quad \left(\frac{\partial v}{\partial r}\right)_{r=b} = 0.$$

Solution:

$$)_{r=a}=V_\infty+\frac{2}{3}\frac{a^3-b^3}{a^2}V_\infty\sum_{n=0}^{\infty}\sum_{\alpha}\frac{(2n+1)\,\alpha^2a^2U_n^2(\alpha a)}{\{\frac{1}{4}+\alpha^2a^2-(n+\frac{1}{2})^2\}aU_n^2(\alpha a)-\{\frac{1}{4}+\alpha^2b^2-(n+\frac{1}{2})^2\}bU_n^2(\alpha b)}P_n(\mu)\,e^{-\kappa\alpha 2t} \qquad (4)$$

α = positive non-zero roots of the equation $R_n'(\alpha b)\,S_n'(\alpha a)=R_n'(\alpha a)\,S_n'(\alpha b)$,

$R_n(x)=x^{-\frac{1}{2}}\,J_{n+\frac{1}{2}}(x)$,

$S_n(x)=x^{-\frac{1}{2}}\,J_{-(n+\frac{1}{2})}(x)$,

$U_n(\alpha r)$ = the spherical shell analogue of the Hankel function.

Note on $U_n(\alpha r)$. A fundamental solution of Equ. (3) is $e^{-\kappa\alpha^2 t}U_n(\alpha r)\,P_n(\mu)$, where $U_n(\alpha r)$ includes Bessel functions of the second kind, as the origin is excluded in the region under consideration. In fact,

$$U_n(\alpha r)=R_n(\alpha r)\,S_n'(\alpha a)-R_n'(\alpha a)\,S_n(\alpha r).$$

Note on equ. (4). When b becomes zero, Problem II becomes Problem I and Equ. (4) becomes identical with Equ. (2).

III. DIFFUSION IN AN INDEFINITELY THIN CORTEX

When the spherical shell in Problem II becomes indefinitely thin, Equ. (4) becomes

$$\left.\begin{aligned}(V)_{r=a}&=V_\infty+V_\infty\sum_{n=1}^{\infty}(2n+1)\,P_n(\mu)\,e^{-n(n+1)\kappa' t},\\ \kappa'&=\kappa/a^2.\end{aligned}\right\} \qquad (5)$$

The experimental conduction velocity curve is conveniently plotted in terms of the angle, subtended at the egg centre, by the affected arc of the cortex (ordinates), against time (abscissae). The theoretical curves for intracellular diffusion, Equ. (2), and intracortical diffusion, Equ. (5), are given in Figs. 1 and 2. In each case a series of theoretical curves is given, each corresponding to a different concentration of the diffusing substance reaching particular points on the cortex and causing the change in cortical structure. In Fig. 1, for example, the curve labelled $V/V_\infty=0{\cdot}4$ is the one appropriate to the condition that the cortical change occurs when the concentration of the diffusing substance reaches the value $V/V_\infty=0{\cdot}4$ at the surface. In the theoretical curves, the abscissae are in the units $\kappa' t=\kappa t/a^2$. To compare the experimental curve with the theoretical ones, t is multiplied by a^{-2} and various values of κ, shown against each experimental curve in Figs. 1 and 2. a is, of course, the radius of the egg.

Figs. 1 and 2 show that the diffusion hypothesis cannot be rejected, and that, as was expected for other reasons, intracellular diffusion is more consistent with the experimental data than cortical diffusion. The best theoretical curve is the one corresponding to a diffusion constant of 0·07 cm.^{2}/day, which suggests that the molecular weight of the diffusing substance may be of the order of 1000. Furthermore, a rather high concentration is needed at the surface for the cortical change to take place. It will be observed that there are discrepancies between the theoretical and the experimental curves. These may be due to errors in measurements, which

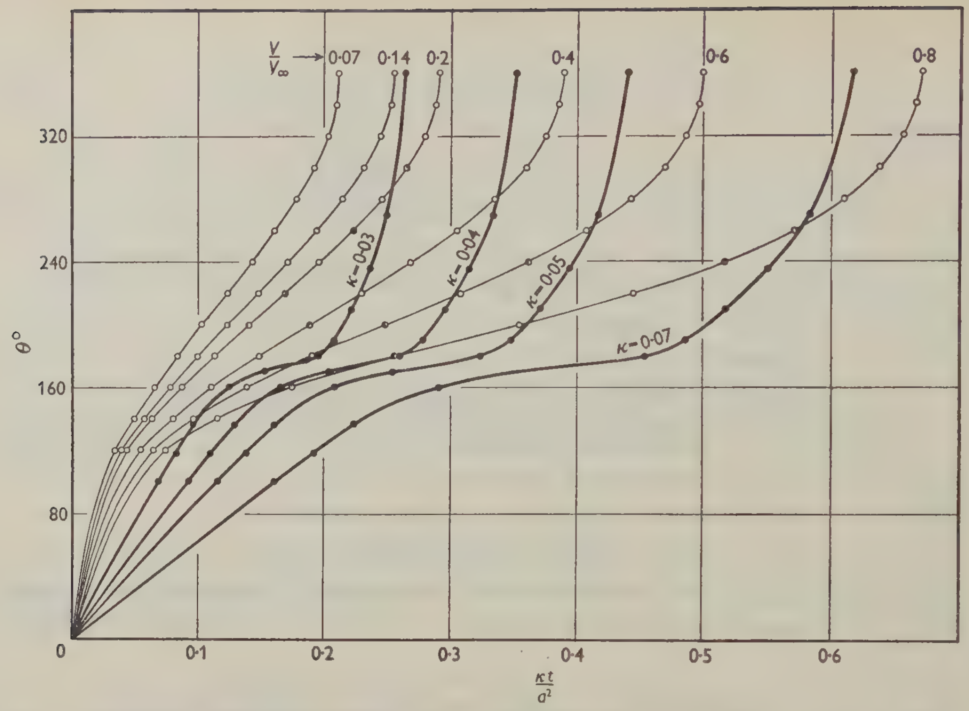

Fig. 1. Intracellular diffusion hypothesis. ○—○, theoretical curves for different values of V/V_∞, the fraction of the final concentration of the diffusing substance which causes the change in cortical structure. At $t=\infty$, $V/V_\infty=1$. ●—●, experimental curves for different values of the diffusion constant, κ, in cm.^{2}/day. Ordinates, angle subtended at centre of sphere by affected part of the cortex; abscissae, $\kappa t/a^2$, where t=time in seconds and a=radius of egg in cm.

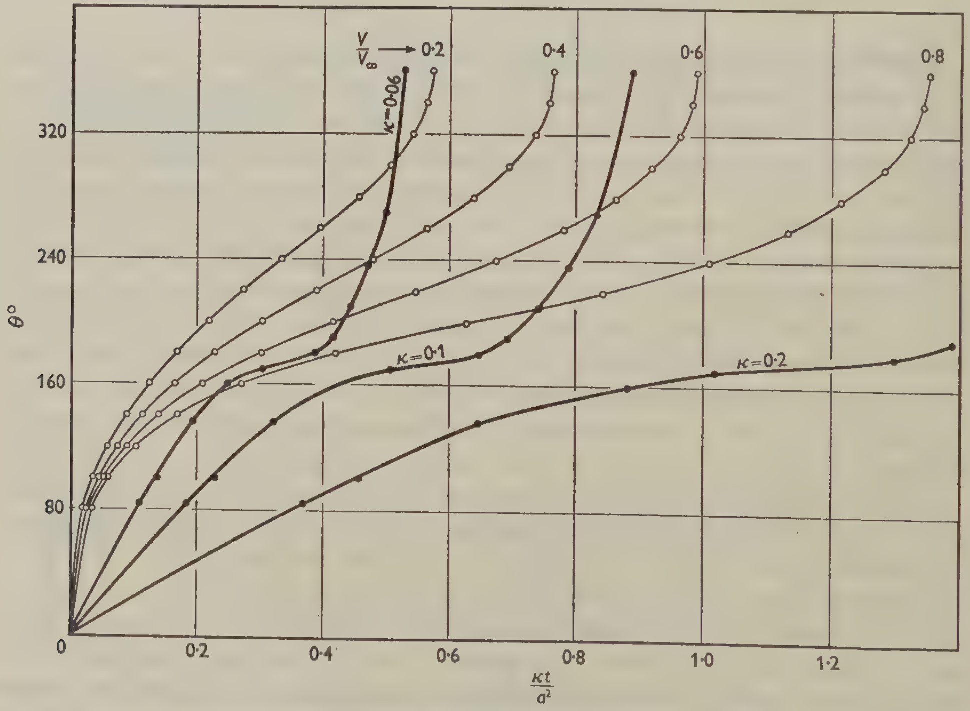

Fig. 2. Intracortical diffusion hypothesis. ○—○, theoretical curves for different values of V/V_∞ in an indefinitely thin spherical shell. ●—●, experimental curves for different values of κ. Ordinates, abscissae and units as in Fig. 1.

present many difficulties, especially in the early stages of the reaction; or they may indicate that the hypothetical diffusion mechanism is over-simplified and requires modification; alternatively, the similarity between the theoretical and experimental curves and the reasonable value for the diffusion constant may be fortuitous. The resolution of these questions requires further experiment.

I am much indebted to Mr H. Ruben for invaluable assistance in solving the diffusion equations.

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IONIC REGULATION IN SOME MARINE INVERTEBRATES

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(*Received* 29 *January* 1949)

I. INTRODUCTION

Much attention has been given in recent years to the study of osmotic and ionic regulation in aquatic animals. Marine invertebrates are generally in osmotic equilibrium with sea water, but some exceptions exist in the Crustacea. Although the integument in whole or part is permeable in some degree to the ions of sea water, the higher invertebrates are able to maintain body fluids differing from the external medium in having higher potassium and calcium and lower magnesium and sulphate concentrations. Krogh (1939) has discussed the literature on these subjects up to 1938.

If ionic regulation is defined as the maintenance in a body fluid of concentrations of ions differing from those of a passive equilibrium with the external medium, few reliable data on this subject exist in the literature. Analyses of invertebrate body fluids are extensive (e.g. Bethe & Berger, 1931; Cole, 1940; Hayes & Pelluet, 1947), but in many cases entirely inadequate on account of the inaccuracy of the methods and procedure used for the determination of inorganic constituents (see Robertson & Webb, 1939). For proper comparisons of equilibria the analyses should be given in parts per unit weight of water (or molality) and not simply on a volume basis which does not take into account the space occupied by the colloids. Few investigators do this and some do not give the chloride content of the sea water with which the animal was in equilibrium. The water content of the blood of some decapod Crustacea and cephalopod Mollusca may be 5–12% less than that of sea water owing to the presence of protein, which sometimes attains a concentration of over 100 g./l. (*Loligo*, *Eledone*). Associated with this, the interpretation of concentration differences is complicated by the Donnan equilibrium and the existence of undissociated compounds of calcium with proteins. The effects produced by the indiffusibility of protein and calcium-protein complexes across gills or other boundary membranes need be considered only in certain invertebrates, not in echinoderms, annelids or bivalve molluscs which have very small amounts of protein in their body fluids. From the data presented in this paper and from Webb (1940), it is conceivable that the blood of a crab could be in equilibrium with sea water without any ionic regulation at all and yet show the following concentration differences, taking sea-water values as 100: Na and K 103, Cl 97, Mg and SO_4 106 and 94 respectively, and Ca 115–120. These differences illustrate the Donnan effect in the blood of a crab with 60 g. protein/l., the calcium figure, in addition, illustrating the indiffusible calcium proteinate fraction.

Other factors which must be taken into consideration in the interpretation of concentration differences are those of electro-neutrality and osmotic pressure. The first of these is self-evident. If by some active process the concentration of sulphate ions in the blood of an animal is reduced, there would require to be an increase in the concentration of the coexisting anions, for example, chloride, to balance the cations. This could be regarded as passive accumulation of chloride brought about by active elimination of sulphate. Similarly, in an ultrafiltrate of plasma, if the concentration of a cation such as magnesium is increased by cell activity to a value much higher than its original concentration in the plasma, the total ions remaining the same, the remaining cations would be reduced correspondingly. The fluid formed by the antennary glands of marine crabs and lobsters, essentially a modified ultrafiltrate, isotonic with the blood but containing higher concentrations of Mg^{++} and $SO_4^=$, shows correspondingly lower values of Na^+, K^+, and Ca^{++}, and sometimes Cl^- ions.

Ion concentration differences may arise between animal body fluids and sea water in osmotic equilibrium as a consequence of deviations from the ideal laws of solutions. Thus the Na^+ and Cl^- ions in a solution of sodium chloride have a relatively greater osmotic effect than the Na^+ and $SO_4^=$ ions in a solution of sodium sulphate. The freezing-point of 0·5 molal-NaCl is about $-1{\cdot}67°$ C., but that of 0·5 M-Na_2SO_4 with its three ions is not $(\frac{3}{2} \times -1{\cdot}67)$ or $-2{\cdot}51°$ C., but about $-1{\cdot}9°$ C. (data calculated from *International Critical Tables*). In a synthetic sea water of $\Delta - 1{\cdot}8°$ C. made up of the usual salts in isosmotic solutions, for example in the case of NaCl 0·54 M and of Na_2SO_4 0·46 M, I have calculated that a reduction of the sulphate concentration to a half if compensated osmotically by increase in the concentration of isosmotic sodium chloride would bring about a fall in the total Na to 97·5 % and an increase of the Cl to 103·2 %. This is simply the result of the replacement of a less effective osmotic substance—sodium sulphate—by a more effective one: sodium chloride. As will be seen below, the composition of *Aurelia* mesogloeal tissue shows somewhat similar features with regard to Na^+, Cl^-, and $SO_4^=$ ions.

This paper contains data on the ionic regulation of certain common marine invertebrates, with some information on the mechanisms concerned. In outlook it is akin to that of Robertson (1939) and Webb (1940).

II. MATERIAL AND METHODS

The animals used were all freshly collected at Millport and were kept for a few days in tanks of gently flowing natural sea water of 17·6–18·1 ‰ chlorinity, the concentration in their normal habitat. A final equilibration period of 24 hr. was given in a glass tank of aerated sea water of the same concentration or within 1 % of that of the main tanks. The sea water temperature was within the range 10–15° C.

Body fluids were obtained in different ways: with a syringe from the body cavity (*Marthasterias* and annelids) or heart (*Pecten*, *Mya*); with a glass cannula from the basal joint of the legs and from the antennary glands (crustaceans); by incisions in the sinuses of the foot (*Neptunea*, *Buccinum*), or the adductor muscles (*Ensis*), allowing the blood to drain; [by incisions in the dorsal surface (Pleurobranchus), withdrawing the blood in a pipette;] with a syringe or pipette from the renal sacs, ventricle, branchial hearts and principal blood vessels (cephalopods), especially the contractile anterior vena cava (*Eledone*) and posterior mantle veins (*Loligo*). When small quantities of blood were required from lobsters, a walking leg was severed about the middle and the fluid allowed to drip into a receptacle. When sufficient blood had been obtained, the leg was removed at the breaking plane. The fluids were immediately chilled, centrifuged and poured off from the corpuscles. Heparin was required only for crustacean blood; sometimes clotting of the plasma took place despite the anti-coagulant, in which case serum was used (*Palinurus*).

Methods of inorganic analysis were those of Robertson & Webb (1939), but a barium iodate method was used for the estimation of sulphate (Webb, 1939*b*). Samples for the metallic radicles were ashed usually in a muffle furnace. All the analytic techniques were rigorously controlled by simultaneous estimations on sea water of known chlorinity. Water contents were obtained by evaporating 1 ml. samples on a water-bath and then drying the residue in an oven at 100–101° C. for 4 hr. With sea water this procedure gave fairly consistent results with no loss of chloride but with a water of crystallization content in the salts of nearly 13%. The water-content values of sea water are therefore about 0·5% too low, but a compensating error probably exists in the corresponding values for the body fluids. Protein was either determined gravimetrically (Robinson & Hogden, 1941), or estimated by subtracting from the total solids the weight of salts found by analysis plus a water of crystallization value similar to that found in the case of sea water.

Simultaneously with the collection of the body fluids, a sample of the sea water was taken and analysed for chloride, its composition being then calculated from the following standard sea-water ratios: Na/Cl 0·5549, K/Cl 0·02009, Ca/Cl 0·02122, Mg/Cl 0·06694, SO_4/Cl 0·1396 (Webb, 1939*a*). Dialysis experiments against sea water were carried out with collodion membranes as described by Robertson (1939), but the sea water was changed several times and had a final pH of 8·0–8·1. In the case of the scyphozoans, echinoderms, annelids, lamellibranchs and tectibranchs, the figures for the dialysed body fluids did not differ from those of the sea water by more than may be accounted for by experimental error. The low protein content, less than 2·5 g./l., accounts for this. In these cases variation from ionic equilibrium was determined by dividing the body fluid values by the more accurate sea-water figures. In general, the latitude of analytical error is such that a true difference is probable only when the original body fluid differs from the dialysis values by the following percentages: Na 0·6, K 2·6, Ca 1·5, Mg 1·8, Cl 1·1, SO_4 1·2. With magnesium and potassium an increased accuracy was obtained by duplicate or triplicate determinations in several instances, for example potassium in *Arenicola*, in which the coelomic fluid and sea-water values were very close.

The concentrations of the individual ions are given on a mg./g. water basis. The differences between these values and the usual weight/volume concentrations may be calculated from the water contents.

III. COELENTERATA

In members of the class Scyphozoa there is a series of canals in which a circulation of sea water takes place, the so-called gastrovascular system. This sea water is perhaps to be regarded as a true body fluid, bringing oxygen and the products of digestion to the endoderm cells of the system and removing waste material. The jelly-like mesogloea between the ectoderm and endoderm is a simple supporting tissue through which must diffuse material necessary for the metabolism of its cells and the cells of the outer ectodermal epithelium. The analyses of the juice obtained by the liquefaction of the bodies of *Aurelia aurita* (var. *flavidula*) and *Cyanea* by Macallum (1903), and of the ash of the central part of the exumbrella of *Aequorea*, *Dactylometra* and *Cyanea* by Koizumi & Hosoi (1936), agree in showing much higher potassium (144–256%) and much lower sulphate (42–88%) than in the surrounding sea water. While sodium, calcium and magnesium showed only minor differences from equilibrium values, chloride was always slightly higher (102–109%). Certain freezing-point determinations of *Aurelia* and *Cyanea* made by Macallum suggested hypertonicity of the tissue juice, but the determinations are not supported by his chemical data, as Bateman (1932) has pointed out, and if true would mean that considerable quantities of organic molecules must be present. Bateman's vapour pressure measurements show almost complete isotonicity between several species of medusae and sea water. Satisfactory agreement in total g.-ions per kg. water content was found between sea water and the three medusae analysed by Koizumi & Hosoi and was found also in the analyses given below. It seems certain that Macallum's freezing-point determinations were in error, since he found a Δ of −1·825° C. for sea water of 16·54 chlorinity, whereas the true value may be calculated to be −1·62° C. (Sverdrup, Johnson & Fleming, 1942, p. 66).

Since at least part of the elevated potassium found by these authors may be attributed to leakage of this ion from cells or to the inclusion of cells in the analyses, further information was desired. If a thin layer including the outer epithelium is removed from the jelly, and then cuts or hollows made in the latter with a scalpel, a clear cell-free fluid collects which may be withdrawn for analysis. Analyses of tissue fluid obtained in this way from two specimens of *Aurelia aurita* are given in Table 1. All the ions differed from those in the surrounding sea water, sulphate especially being conspicuously low, but the striking accumulation of potassium shown by previous investigators is absent. It did not exceed 108% of the value in

Table 1. Aurelia aurita

	mg./g. water						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
Mesogloeal tissue								
Fluid I	10·45	0·411	0·390	1·235	19·65	1·458	0·4	981
Fluid II	10·42	0·397	0·383	1·240	19·82	1·043	0·9	981
I after dialysis	10·59	0·380	0·408	1·270	19·00	2·642	0·4	981
II after dialysis	10·52	0·385	0·406	1·251	19·08	2·656	0·9	981
Sea water	10·55	0·382	0·404	1·273	19·02	2·655	—	983

sea water, and all the other ions except sulphate were much closer to equilibrium. The most abundant ions, sodium and chloride, were respectively 1 % below and 3–4 % above a static equilibrium, while the alkaline earth cations fell short by 3–6 %.

In considering this peculiar ionic regulation, it is obvious that no part is played by the small concentration of protein. Indeed, except for potassium and sulphate, even the direction of the values of the other ions relative to sea water is opposite to that of a Donnan equilibrium. The total concentration of ions in the first and second specimens was 1·093 and 1·094 g.-ions/kg. water, compared with 1·095 for sea water (1·097 including bicarbonate), and the concentration of cations (milli-equivalents) checked with that of the anions within 0·7 % in each case. Nevertheless, the individual ion concentrations are such as to provide evidence of an equilibrium maintained by the expenditure of energy, that is, a steady state. An active elimination of sulphate ions with associated cations may be postulated to take place at the outer ectodermal and inner endodermal epithelia, and, granted this assumption, there would be a fall in the cation concentration in order to maintain isotonicity, owing to the greater osmotic effect of solutions of the common monovalent ions compared with solutions containing divalent ions, as pointed out earlier. While this explanation would account in the main for the lower concentrations of Na^+, Ca^{++} and Mg^{++}, a further assumption of active uptake of K^+ by the cells bounding the mesogloea must be made to produce a higher concentration of this ion. No such active transfer is necessary for the higher Cl^- concentration, since active elimination of $SO_4^=$ anions would necessitate an increase in the Cl^- anion to balance the cations. Indeed, the earlier calculation of an increase of Cl^- to 103·2 % if the $SO_4^=$ is halved is closely approached in both specimens, the first with 103·3 % Cl^- and 54·9 % $SO_4^=$, the second with 104·2 % Cl^- and 39·3 % $SO_4^=$.

It may be concluded that in this member of a primitive class, the ionic composition of the internal jelly-like medium is controlled by its bounding layers of cells. The assumptions concerning the mechanism of control may not be wholly valid. Analyses of the fluid in the gastrovascular system may provide further clues. It should be remembered that a few fresh-water medusae exist in which the bounding epithelia must control both salt concentration and uptake of water.

IV. ECHINODERMATA

The analyses of Koizumi (1932), Bialaszewicz (1933) and Cole (1940) all show that the concentration of ions in the coelomic fluid of asteroids, echinoids and holothurians is very similar to sea water. A tendency for slightly increased potassium and lower magnesium is evident, the differences usually being within about 8 and 5 % respectively. These differences are probably significant in some cases, despite the fact that the sea-water ionic values given by these authors may differ from the true values by similar or even greater amounts. Robertson (1939), however, found no significant regulation in the case of *Echinus* body fluid.

Analysis of the perivisceral fluid of a large specimen of the spiny starfish *Marthasterias glacialis* showed it to be closely similar to sea water in ionic composition (Table 2). Regulation of potassium is evident, however, and perhaps of magnesium.

Table 2. Marthasterias glacialis

	mg./g. water						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
Perivisceral fluid	10·57	0·425	0·408	1·244	19·18	2·650	0·6	983
Fluid after dialysis	10·51	0·379	0·407	1·273	19·05	2·650	0·6	982
Sea water	10·56	0·382	0·404	1·274	19·03	2·546	—	983
Body fluid values as percentage of sea water values	100·1	111·2	101·0	97·7	100·8	99·8	—	—

After a further 8 days, the potassium had fallen to 103% of the sea-water value, and 18 days after the first analysis was still at this figure. At this stage fluid from the water vascular system was obtained by collecting it as it flowed from the cut ends of tube-feet, and analysed together with the perivisceral fluid. Both fluids contained corpuscles which were removed. Within the limits of error, both agreed in their sodium, calcium, chloride and sulphate content, but potassium was as much as 11% higher in the ambulacral fluid. This is a peculiar finding in a fluid usually regarded as practically unmodified sea water. Bethe & Berger (1931) claimed a much higher potassium content in the water vascular system of *Echinus* compared with the coelomic fluid. This was reinvestigated in a mature *E. esculentus*. After dissecting out gut and gonad and cleaning the inner part of the shell, orange-yellow water vascular fluid was collected by pressing it out of the tube-feet ampullae. After filtration to remove corpuscles, analysis of its potassium content showed a value 118% of that of sea water, compared with a corresponding one of 107% for the perivisceral fluid. Thus the value in the water vascular fluid was 11% higher than that in the perivisceral fluid, a finding similar to that in *Marthasterias*. Ionic regulation of potassium was not found by Robertson (1939) in *Echinus*. This discrepancy may perhaps be linked with the reproductive state of the animal: in the present case its gonad was ripe (analysed 20 March), in the previous instance spawning was long past (analysed in September).

Explanation of this potassium regulation is difficult and can be but speculative. The high value in the water vascular system may possibly be attributed to active absorption of this ion from sea water by the walls of the tube-feet, associated in some way with a high requirement for potassium by the muscles of the tube-feet and their ampullae. High values of potassium in the perivisceral fluid may be due simply to the diffusion of this ion into it from the water vascular system. Further outward diffusion to the surrounding sea water through the gills of the dorsal surface (*Marthasterias*) or through the gills of the lantern coelom (*Echinus*) would lower the potassium content to an intermediate value. On the other hand, the gills may be the seat of absorption of this ion from sea water, and whether one of the processes or both are responsible for regulation in the perivisceral fluid is unknown.

V. ANNELIDA

A few analyses of marine worms have been published by Cole (1940), Bialaszewicz (1933) and Bethe & Berger (1931) indicating that potassium is accumulated (*Arenicola*, *Amphitrite*, *Aphrodite*, *Echiurus*) and magnesium sometimes reduced (*Echiurus*, *Sipunculus*) in their body fluids. Bialaszewicz also finds in the Naples Aquarium an increase of 20% in the calcium concentration and a decrease of 8% in sulphate in *Arenicola claparedii*, and a small decrease in the sulphate of *Aphrodite*, compared with sea water.

Coelomic fluid from a specimen of *Aphrodite aculeata* and from ten individuals of *Arenicola marina* were both found to be in equilibrium with the surrounding sea water except in the case of potassium ions in the former and potassium and sulphate ions in the latter (Table 3). The specimen of *Aphrodite* was mature with male germ cells in the coelomic cavity. Its protein content was low, only about 1 g./l., but *Arenicola* had an even lower figure. Numerous confirmatory analyses of sulphate established its reduced value in the body fluid of the lugworm, a value which on dialysing the fluid rose to equal that of the sea water. The slightly increased potassium of *Arenicola* confirmed in other specimens, is also the result of ionic regulation, since it, too, was abolished after dialysis against sea water. The mechanism of this ionic regulation is unknown. It may be a consequence of selective activity by the 'nephridia' (= nephromyxia) and control of the absorption of these ions by the body wall, the former organs execreting a fluid richer in sulphate and poorer in potassium ions than the coelomic fluid. An attempt to test this hypothesis was made by ligaturing worms behind the sixth pair of nephridia, anticipating that the posterior part of the worm without these organs might come to show a sulphate concentration similar to sea water. However, the occlusional haemorrhage which resulted from the ligature, and the doubt whether separation was complete, make one diffident in accepting the results—identical sulphate values in both regions—at their face value. The hypothesis is still not proved.

Table 3. *Polychaeta*

Coelomic fluid	Concentrations as percentage of sea-water values						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
Aphrodite	99·4	126·0	100·0	98·8	100·3	99·9	0·9	981
Arenicola	100·1	103·5	99·8	100·3	99·7	92·2	0·2	983

Schlieper (1929) found *A. marina* from natural habitats of full strength and 44% sea water to have body fluids isosmotic with the external medium. In an experiment with 16% sea water, the body fluid attained osmotic equilibrium in 26 hr. Beadle's vapour pressure measurements given by Wells & Ledingham (1940) indicate approximate equilibrium with 25% sea water in about 7 hr. But this osmotic equilibrium in dilute sea water does not necessarily imply ionic equilibrium. The coelomic fluid of fourteen worms collected at Millport after a day of rain had a chloride content of 13·1 mg./g. water. Compared with sea water of the same chlorinity, the percentages of the ions were as follows: Na 100%, K 120%, Ca 112%, Mg 99%, Cl 100%, and SO_4 95%. Differences between the percentages of potassium and calcium in these worms and those in full strength sea water (Table 3) prompted an experiment in which three specimens were kept for 23 hr. in 75% and 15 hr. in 50% sea water. The worms increased in weight by 28%. Total base, estimated by Hald's (1934) method, and chloride were identical in the pooled coelomic fluid of these individuals and in the dilute sea water, but the other ions analysed, expressed as percentages of the external medium, were as follows: K 118%, Ca 113%, SO_4 90%. Close agreement is apparent in the potassium and calcium values found in this experiment and in the fourteen worms from dilute sea water. Whether this apparent increased ionic regulation of potassium and regulation of calcium in dilute media may be taken as the result of increased activity of the mechanism responsible for regulation in full-strength sea water is uncertain. The high values may be due partly to the slower acclimatization of the tissues to the reduced salinity, calcium and potassium being lost from the cells to the coelomic fluid at a rate greater than can be offset by diffusion of these ions between the coelomic fluid and sea water.

The use of sea water or artificial sea water as a bathing medium for experiments on *Arenicola* tissues seems to be justified by the ionic composition of the coelomic fluid of worms in full-strength sea water, but dilute sea water as a tissue medium for testing the physiological effects of a hypotonic environment may not be quite equivalent to a medium based on the somewhat different composition of the coelomic fluid during acclimatization (cf. Wells & Ledingham, 1940, 1942).

VI. ARTHROPODA

Increased values of sodium, potassium and calcium, and lowered values of magnesium and sulphate have been found in the blood of the species of marine decapod Crustacea hitherto examined. The mechanism of this ionic regulation seems to be as follows: active absorption by the gills of sodium, potassium and calcium against a concentration gradient favouring their loss by outward diffusion; inward diffusion of magnesium and sulphate in accordance with the concentration gradient; differential excretion by the antennary glands tending to eliminate magnesium and sulphate from the blood on the one hand, and to conserve sodium and potassium on the other; active absorption of water (Robertson, 1939; Webb, 1940).

Analyses of *Nephrops norvegicus*, *Lithodes maia* and *Palinurus vulgaris*, all male specimens, are presented in Table 4. The composition of the plasmas falls into line with previous analyses of other genera, the cations except magnesium being higher, and magnesium and sulphate lower, than the corresponding sea-water values. Potassium concentration is lower in *Nephrops*. After dialysis, a small Donnan effect is apparent, the cations of the plasma exceeding and the anions falling below their concentrations in sea water. It is obvious that there is active ionic regulation in these three decapods. The existence of indiffusible compounds of calcium with protein is indicated by the high concentration of this radicle in the dialysed plasmas of the three decapods. Such compounds seem to be a general feature of the protein-rich crustacean plasma: by dialysis they have been shown to exist in *Ligia* (Numanoi, 1937), *Sesarma* (Numanoi, 1939), *Cancer*, *Homarus* (Robertson, 1939) and *Carcinus* (Webb, 1940), and, using ultrafiltration technique, in *Maia* (Bialaszewicz, 1932) and *Carcinus* (Robertson, 1937).

Table 4. Lithodes, Nephrops *and* Palinurus

	mg./g. water						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
Lithodes								
Original plasma	10·96	0·488	0·495	1·269	19·03	2·470	38	954
Plasma after dialysis	10·56	0·382	0·440	1·311	18·48	2·563	38	954
Nephrops								
Original plasma	11·90	0·296	0·555	0·216	18·40	1·780	33	961
Plasma after dialysis	10·54	0·384	0·448	1·297	18·59	2·572	33	961
Sea water	10·43	0·378	0·399	1·258	18·80	2·624	—	983
*Palinurus**								
Original serum	12·52	0·404	0·539	0·404	19·74	2·046	49	945
Serum after dialysis	11·33	0·411	0·500	1·378	19·57	2·779	49	945
Sea water*	11·01	0·399	0·421	1·329	19·85	2·771	—	983

* Analysed at Marine Biological Station, Port Erin.

Table 5. *Antennary gland secretion*

	Concentrations as percentage of blood values					
	Na	K	Ca	Mg	Cl	SO_4
Nephrops						
Secretion	98	83	81	130	101	106
Ultrafiltrate of blood*	99	99	89	97	101	103
Homarus						
Secretion	99	91	64	180	101	159
Ultrafiltrate*	99	99	87	97	101	103
Palinurus						
Secretion	98	65	86	137	101	98
Ultrafiltrate*	98	98	84	96	102	104

* Calculated from dialysis experiments, using the mean Donnan ratio except in the case of calcium.

In Table 5 the ionic composition of the antennary gland secretion of specimens of *Nephrops*, *Palinurus* and *Homarus vulgaris* is compared with that of an ultrafiltrate of the blood plasma. The differential excretion in *Nephrops* and *Homarus*, eliminating magnesium and sulphate while conserving sodium, potassium and calcium, is in line with the composition of the blood. The same is true of *Palinurus*, except for the slightly lower sulphate in the secretion. With respect to magnesium and sulphate, differential excretion in *Homarus* is exceeded only by *Carcinus* (Webb, 1940). Forster & Zia-Walrath's (1941) experiments with injected inulin and creatinine, in which there was identity of concentration of these two substances in the plasma and antennary gland fluid of *Homarus americanus*, indicate that water is not reabsorbed after filtration. The alternative but improbable view that the high magnesium and sulphate concentrations of the excreted fluid are due not to active secretion of these ions but to absorption of water by the gland epithelium is therefore untenable. The analyses presented in Table 5, taken in conjunction with those on *Carcinus* (Webb, 1940), *Cancer* (Robertson, 1939), and *Maia* (Bialaszewicz, 1932), leave no doubt that the antennary glands play a very important part in ionic regulation in the marine decapod Crustacea.

A comparison of ionic regulation in members of the Decapoda which have been analysed and compared using similar chemical and dialysis methods is given in Table 6. The general picture is the same in the four groups, a raised content of sodium and calcium in the blood, usually a raised potassium, and lower values of magnesium and sulphate, with the chloride ion very close to the value expected if it were in dialysis equilibrium with the external medium. Departure from equilibrium is greatest perhaps in the two genera of Astacura, especially in their very low magnesium values, least in *Lithodes* and *Cancer*. Somewhat puzzling have been the concentrations of blood potassium. Values considerably lower than that in sea water have been found in *Nephrops* and sometimes in *Homarus*, contrasted with previous higher values in *Homarus*,* *Cancer* (Robertson, 1939) and *Carcinus* (Webb, 1940). In five specimens of *Nephrops* examined potassium was only 71–89% of the sea-water value, in four specimens of *Homarus* 74–102%. The figure for *Palinurus* in Table 4 is the mean of values 97% and 106%, the latter being an analysis of the same individual 4 days after the first. Such variations have not yet been interpreted. A possible basis may be alterations in the muscle cell—blood plasma steady state with respect to potassium and sodium ions. Perhaps there is a physiological association between the low magnesium and the reduced potassium of these three species. It should be noted that where concurrent analyses of potassium were made on the secretion of the antennary glands in the genera of Astacura, the values were 81–92% of the actual concentrations in the plasma, indicating that the glands were tending to conserve potassium and thereby elevate the blood values.

Table 6. *Ionic regulation in the decapod Crustacea*

	Concentrations in plasma as percentage of concentration in dialysed plasma						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
Anomura								
Lithodes	104	128	113	97	103	96	38	954
Astacura								
Nephrops	113	77	124	17	99	69	33	961
*Homarus**	110	85	131	14	101	32	29	964
Palinura								
Palinurus	111	98	108	29	101	74	49	945
Brachyura								
*Cancer**	109	115	118	44	98	99	37	961
Carcinus†	110	118	108	34	104	61	55	936

* Robertson (1939) and further analyses. † Webb (1940).

The crustaceans in Table 6 may be arranged in the series *Lithodes*, *Cancer*, *Carcinus*, *Palinurus*, *Nephrops* and *Homarus* in which the magnesium concentration

* The accuracy of a high value of potassium found in *Homarus* was questioned in a footnote.

falls from 97 to 14% of the values in equilibrium with sea water. There is a fairly good inverse relationship here between active species and magnesium content of the blood, the slow-moving *Lithodes* and *Cancer* with their relatively high magnesium contrasting with the more active *Carcinus*, *Nephrops*, *Homarus* and *Palinurus* in which magnesium is 14–34% of the equilibrium values. Osmotically, the reduction of magnesium ions is more than counterbalanced by the increase in sodium ions. For example, a decrease of 40 mM Mg is offset by an increase of 52 mM Na in *Palinurus*.

While the fact of ionic regulation is established, many points about its mechanism are obscure. One such point is the differential excretion and retention of ions in the secretion of the antennary glands. Forces tending to keep constant the total concentration of ions seem to be at work here. If the concentration of Mg^{++} and $SO_4^{=}$ ions in the secretion is increased by the active work of the epithelium somewhere in the organs, corresponding reductions in the other cations and anions are necessary to maintain isotonicity. Such reductions are evident in Table 5, but they are not proportionally the same, and it seems that some active regulation of each ion by the epithelium must be postulated for the fine adjustment, as it were, of the composition of the fluid. Again, consideration of the composition of the blood from the point of view of total concentration shows the necessity for increases in the other ions where Mg^{++} and $SO_4^{=}$ are reduced. This may be regarded as the coarse adjustment of the blood composition in such cases, the mechanism of fine adjustment being located at the epithelia of the antennary glands and outer integument.

The absorption of water and ions to compensate the loss from the antennary glands has not been studied. Inward diffusion of Mg^{++} and $SO_4^{=}$ ions from sea water may be sufficient to balance the loss of these ions except perhaps in *Lithodes* where the gradient is very low. The epithelium of the gills must co-ordinate the active uptake of Na^+, K^+, Ca^{++}, Cl^- and water with this diffusion, in order to maintain the total ion concentration of the blood at a constant level. It is possible that some of the uptake of Na^+, K^+, and Cl^- against a concentration gradient is due not to cellular accumulation of these ions but to the requirements of constant total concentration in the fluid replacing the excreted salts and water. Assuming that there was an overall balance in gain and loss of Mg^{++} and $SO_4^{=}$ ions in these crustaceans, this fluid would have a concentration of the remaining ions higher than in sea water, in order to balance the lower concentrations of Mg^{++} and $SO_4^{=}$. But this would not be the case in *Carcinus*, which excretes Mg^{++} and $SO_4^{=}$ in concentrations superior to those in sea water (Webb, 1940).

VII. MOLLUSCA

Previous analyses of molluscan blood present a sorry spectacle of disagreement. Kumano (1929) found almost complete agreement between blood, pericardial fluid and sea water in the case of *Ostrea circumpicta*, except for magnesium and sulphate. These ions are given as, respectively, 11 and 7% higher in the blood than in sea water, while the corresponding values in the pericardial fluid are 5% lower and 2% higher. *Pinna nobilis*, a lamellibranch analysed by Bialaszewicz (1933) showed higher values of potassium and calcium, and slightly lower magnesium. Bethe & Berger (1931) give figures showing higher sodium and potassium in the gastropods *Aplysia* and *Doris*, and lower values of these ions in *Mytilus*. Magnesium is shown as above sea-water level in *Doris* (123 %), about the same level in *Aplysia* (98 %), with calcium slightly higher in all three. Krogh's (1939, p. 60) analysis of *Mytilus* differs in its high potassium concentration, 175 %, and he gives a sulphate concentration of 87 %. Another lamellibranch, *Venus mercenaria*, is stated by Cole (1940) to have the following blood concentrations as compared with sea water: Na 110 %, K 93 %, Ca 209 %, Mg 93 %, Cl 117 %, and SO_4 116 %. Such latitude does he attribute to experimental error and individual variation that he concludes that a uniform distribution of ions exists between the internal and external media in this case!

Hayes & Pelluet (1947) have offered analyses of the bloods of *Pecten*, *Aplysia* and two cephalopods, and very incomplete figures for five other marine molluscs. *Aplysia* and *Pecten* show higher calcium and lower magnesium, *Pecten* in addition having higher potassium (132 % of sea-water value). *Eledone* and *Sepia* show lower sodium and chloride, but higher calcium, and a sulphate figure 151 % that of sea water is given for the lesser octopus. They conclude from their own and previous analyses that no difference exists between inner and outer concentrations of sulphate in the three classes, or of sodium and potassium in lamellibranchs and gastropods, but that regulation is shown in all classes by the higher calcium and lower magnesium figures, and additionally in cephalopods by lower concentrations of sodium and chloride, and higher potassium.

The discrepancies revealed in this survey are attributed by the present writer not in the main to variations in a species or group but predominantly to faulty chemical technique, and partly in some instances to incomplete equilibration of the animals with sea water of known chlorinity. The practice of using only one precipitation in the estimation of calcium, giving falsely high values owing to the contamination of the precipitate with occluded sodium and magnesium (see Robertson & Webb, 1939), is not adequate for solutions such as sea water. Magnesium determinations in the filtrate from the calcium estimations will accordingly be below the true values. The poor performance of the conventional biochemical methods as applied to sea water by the above authors may be illustrated by the K/Cl and Mg/Cl ratios calculated from their figures. These ratios are generally accepted as 0·0201 and 0·067 (Webb, 1939*a*; Sverdrup *et al.*, 1942), but the former varies from 0·0184 (Kumano), 0·0191 (Cole), to 0·0217 (Bialaszewicz and Krogh), capped by ratios of 0·0174 and 0·0286 in a single paper (Bethe & Berger), while the Mg/Cl ratio may be much too small, as shown by 0·047 (Cole) and 0·059 (Bethe & Berger, Hayes & Pelluet). It is not safe to assume that inherent defects of a method are cancelled out in the comparison of body fluids with sea water, since slight changes in technique and in the relative proportions of the various ions may result in different errors in the two cases.

The data in Table 7 have been accumulated by using chemical methods of known accuracy on animals which were in equilibrium with the surrounding sea water. In

Table 7. *Ionic regulation in the Mollusca*

	Concentrations in plasma as percentage of concentration in dialysed plasma						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
Lamellibranchia								
Pecten maximus	99·7	129·9	102·5	97·3	100·0	96·5	2·6	983
Mya arenaria	100·8	106·7	106·6	99·0	99·9	101·0	1·3	982
Ensis ensis	98·7	155·2	107·6	98·7	99·2	86·5	4·4	981
Gastropoda								
Neptunea antiqua	100·6	113·9	101·7	101·1	100·9	97·8	24·1	968
Buccinum undatum	97·3	141·9	104·3	102·8	99·6	90·0	25·3	966
Pleurobranchus membranaceus	100·1	117·1	111·7	99·4	99·6	101·7	0·3	980
Cephalopoda								
Eledone cirrosa	97·4	152·1	107·1	102·7	101·7	77·2	105·0	896
*Loligo forbesi**	95·2	219·4	101·6	101·7	103·5	29·3	149·7	866
Sepia officinalis	97·6	175·4	94·3	—	101·0	80·7	67·6	925

* Analysed immediately without equilibration period.

the case of the lamellibranchs, blood was obtained from twelve specimens of *Mya*, five of *Ensis*, and nine of *Pecten*; in the latter separate analyses were made of two single individuals and groups of two and five to study the variation, but the figures given are based on the weighted means. Features of the class are accumulation of potassium and calcium, virtual equilibrium between the outer and inner concentrations of sodium and chloride since their values in the dialysed plasmas and in sea water were almost identical, and the very small concentration of protein. In *Pecten* and *Ensis* a very slight elimination of magnesium has occurred together with a rather greater diminution in the sulphate concentration. The range of variation in *Pecten* was Na 99–100 %, K 114–160 %, Ca 97–104 %, Mg 96–100 %, Cl 99–101 %, SO_4 94–100 %.

Of the three gastropods examined, *Pleurobranchus* is a tectibranch with no external shell, and the other two are closely allied, belonging to the Prosobranchia. *Buccinum* and *Neptunea* have haemocyanin in their blood, giving a considerably higher total protein content than *Pleurobranchus* and the lamellibranchs. Ionic regulation consisting of increased potassium and calcium is evident in the two prosobranchs, of which five specimens were analysed in each case. The same two ions exceed the equilibrium values in *Pleurobranchus*, in the analysis of which four specimens were used. The extent of variation in potassium was 111–115 % for *Neptunea*, 133–153 % for *Buccinum*, and 114–120 % for *Pleurobranchus*, while that in calcium was 101–103 %, 102–106 %, and 105–118 % respectively. With regard to the predominant sodium and chloride ions, it is seen from Table 7 that they have the same values as they would have under conditions of static equilibrium, except, apparently, sodium in the plasma of *Buccinum*. This lower value is associated with high potassium and a slightly increased magnesium concentration, and it may be the result of forces regulating the total concentration of ions. Sulphate is eliminated to a small extent in *Buccinum*.

Three members of the highly organized Cephalopoda have been examined, the lesser octopus, the squid and the cuttlefish. Analyses of three female *Eledone*, three male *Loligo* and a *Sepia* agree in demonstrating that regulation extends to all the ions. Potassium is much above the equilibrium value, magnesium and chloride only slightly, while sodium and sulphate are below. The potassium concentrations are higher than in other members of the Mollusca, and higher than in any of the analyses of other groups presented in this paper. Range of variation as determined in separate analyses of *Eledone* was Na 96–99%, K 138–174%, Ca 105–108%, Mg 97–108%, Cl 101–102%, SO_4 67–87%. The very high concentrations of protein, mainly haemocyanin, are a conspicuous feature of the group. A value such as that in *Loligo* is about double that of man. It is probable that the sodium and chloride figures, as in *Aurelia*, are related to the osmotic pressure of the blood, sodium and chloride ions replacing the much less effective sodium and sulphate ions, and some of the sodium also being replaced osmotically by potassium, calcium and magnesium ions. With regard to the first point, relevant to sulphate in *Loligo*, it may be calculated that in a synthetic sea water in which three-quarters of the sodium sulphate is replaced by isosmotic sodium chloride, the sodium concentration would fall 3·6% and the chloride increase 4·9%.

Taking the Mollusca as a whole, it is evident that high potassium concentrations are found in the more active species, *Pecten* which swims by clapping its valves together, the specialized burrower *Ensis* with its remarkable mobile foot, and the Cephalopoda. *Buccinum* is an unexplained exception, as it appears to be as equally inactive as *Neptunea*.

Questions arise regarding the mechanism of ionic regulation. How are the concentration differences brought about in this group? The heart in molluscs is surrounded by a coelomic space, the pericardium, in communication with which are the pair of excretory tubules opening externally into the mantle cavity. It seems that there is filtration of fluid through the wall of the heart, and that this fluid together with the secretion of the pericardial glands passes into the tubules. To it will be added the secretion of the tubules themselves, and the final composition of the fluid would depend on how far such processes as secretion and reabsorption of ions take place in the epithelium of the tubule. In analyses I have made of *Pecten*, the chloride and sulphate concentrations of the pericardial fluid were identical with those of the blood, supporting the filtration theory. Unfortunately, excretory fluid is almost impossible to collect from the ends of the tubules, since there is no real reservoir. In one specimen the small amount of fluid obtained was analysed for potassium and sulphate and no differences were found between it and the blood. However, it may have been recently filtered fluid. The cephalopods offered a more promising line of attack, as small amounts of fluid were usually present in the renal sacs. It must be admitted that the precise origin of this fluid is uncertain. In these cephalopods a protein-free fluid probably filters across the walls of the branchial hearts, auricles and ventricle into the pericardial coelom which is in communication with the paired renal sacs in *Eledone* and a single renal sac in *Sepia*. To it will be added the secretion of the renal epithelium which covers the walls of the veins lying within the renal chambers. The walls of the venae cavae and veins joining them in this region are produced into the conspicuous renal appendages, covered with a glandular coelomic epithelium believed to be excretory (Isgrove, 1909; Tompsett, 1939).

Table 8. *Execretory fluids of* Eledone *and* Sepia

	Concentrations as percentage of blood values					
	Na	K	Ca	Mg	Cl	SO_4
Eledone						
Renal sac fluid	102	90	87	89	97	136
Ultrafiltrate of plasma*	99	99	92	98	101	102
Sepia						
Renal sac fluid	—	—	87	—	96	117
Ultrafiltrate*	—	—	89	—	101	102

* Donnan ratio obtained from dialysis experiments, calcium ratio sometimes by ultrafiltration (Greenberg & Gunther, 1930).

Table 8 shows the data obtained in three comparisons of fluid collected from the renal sacs and plasma in *Eledone*, and one in *Sepia*. It is clear that this fluid is far from being an ultrafiltrate of the blood, although it is almost protein-free. In *Eledone* resorption of potassium, calcium, magnesium and chloride, and secretion of sulphate and sodium have taken place, with the result that the level of the former ions in the blood tends to be raised, the level of the latter ions lowered. *Sepia* exhibits the same features as far as the analyses go. The parallel between these analyses and those of the antennary gland secretion is close, and suggests that the mechanisms of ionic regulation in the two groups are essentially similar: the elimination of a fluid in which the ions have undergone differential retention and excretion, balanced by absorption of a similar quantity of fluid in which the concentration of ions is such as to maintain a constant osmotic pressure in the blood. Nothing seems to be known of the rate at which filtration proceeds in the cephalopods: this would give additional information about the importance of the execretory tubules in the salt and water balance of these molluscs. Picken's (1937) data on fresh-water *Anodonta* imply a filtration of six times the animal's weight per day. Such extraordinary water flux is unlikely to obtain in marine lamellibranchs, far less so in cephalopods, but nothing further can be said at present owing to lack of relevant data.

Regarding the assumed absorption of ions and water through the gills and any permeable portions of the external surface, no experiments have been made, but it is apparent that potassium, magnesium, chloride, and usually calcium must be absorbed against the concentration gradient. Sodium and sulphate ions will presumably enter the blood in accordance with the diffusion gradient, but whether this physical process is sufficient to compensate completely for the loss of these ions in renal excretion remains uncertain. The following considerations are relevant to the uptake of water in cephalopods: Botazzi (1908) found the blood of *Octopus vulgaris* to be practically isosmotic with sea water; in *Eledone cirrosa* I have found the vapour pressures of plasma and the surrounding sea water to be identical, using Walker's (1930) modification of Barger's capillary method; the means of the total ionic concentrations of plasma both in *Eledone* and *Loligo* are within 1 % of those in their respective sea waters; although there is a high concentration of protein, the colloid osmotic pressure of cephalopods is only 3–4 cm. water,* according to Meyer (1935). It is unlikely, therefore, that water absorption is wholly due to osmosis.

How far intake of fluid through the gut contributes to the salt and water balance of molluscs is unknown. It may play some part, but information on whether molluscs drink sea water to any extent is lacking. Bethe (1934), however, in experiments in which the mouth was ligatured, clearly showed that the integument of *Aplysia* was permeable to the ions of sea water, and it seems justifiable to conclude that the inward passage of ions and water in relation to ionic regulation takes place chiefly across the gills and outer surface.

VIII. DISCUSSION

The data presented show that ionic regulation is a universal phenomenon in marine invertebrates, differing only in extent among the various groups. In the morphologically rather primitive, relatively inactive, coelenterates, echinoderms, polychaete worms, lamellibranchs and gastropods, there is an accumulation of potassium and calcium, slight in the case of calcium, and a small diminution of sulphate, except in *Aurelia* where it is halved. Sodium and chloride are essentially in equilibrium across the boundary walls of these animals, while any elimination of magnesium is slight. Contrasted with this we have the decapod Crustacea and the Cephalopoda, active groups showing pronounced regulation, extending apparently to all the ions in the blood. In the decapod Crustacea there is the remarkable reduction of magnesium from almost complete equilibrium with the sea-water concentration in the anomuran *Lithodes* to the low values of between one-third and one-seventh in *Carcinus* and the astacuran and palinuran genera, a reduction which is correlated with increased general activity. Sulphate is reduced markedly, but not to the same degree as magnesium. These decapods also show accumulation of calcium, and usually potassium. In addition, they accumulate sodium ions, a feature not found in the other marine invertebrates, but correlated with reduction in magnesium.

Excretory organs play an important part in regulating the composition of the blood in both the crustaceans and cephalopods. By forming fluids differing from ultrafiltrates in their composition by retention and excretion of particular ions, they are tending to alter the levels of these ions in the blood. While magnesium and sulphate are excreted in excess in the crustaceans, which have corresponding lower values of these ions compared with sea-water equilibrium values, sodium and sulphate are eliminated in the cephalopods, which have corresponding lower sodium and sulphate in their plasmas.

Regarding uptake of water and ions in these two groups, there is need for 'balance' experiments, in which the gross excretion or absorption of ions over a period is related to the quantity of ions passed out by the excretory organs. It may be possible to find out whether the alimentary canal is aiding the gills or other permeable portions of the integument in the uptake.

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* These pressures may be too low. Picken's (1936) figures for colloid osmotic pressure in crustaceans are three times those of similar values given by Meyer.

Table 9. *Relative ionic composition of body fluids*

	Weight units (g.)						Equivalents		
	Na	K	Ca	Mg	Cl	SO_4	$\frac{Na+K}{Ca+Mg}$	$\frac{K}{Mg}$	$\frac{Ca}{Mg}$
Sea water	55·5	2·01	2·12	6·69	100	14·0	3·8	0·09	0·19
Aurelia	53	2·05	1·96	6·3	100	6·3	3·8	0·10	0·19
Echinus	55	2·09	2·15	6·6	100	14·0	3·8	0·10	0·20
Marthasterias	55	2·22	2·13	6·5	100	13·8	3·8	0·11	0·20
Arenicola	56	2·09	2·12	6·7	100	12·9	3·8	0·10	0·19
Aphrodite	55	2·52	2·12	6·6	100	13·9	3·8	0·12	0·20
Pecten	55	2·61	2·18	6·5	100	13·5	3·8	0·13	0·20
Mya	56	2·15	2·26	6·6	100	14·1	3·8	0·10	0·21
Ensis	55	3·14	2·30	6·6	100	12·2	3·8	0·15	0·21
Pleurobranchus	56	2·36	2·38	6·7	100	14·3	3·7	0·11	0·22
Neptunea	55	2·30	2·25	6·8	100	13·1	3·7	0·11	0·20
Buccinum	54	2·88	2·31	7·0	100	12·6	3·5	0·13	0·20
Eledone	54	3·07	2·46	7·0	100	10·5	3·5	0·14	0·21
Sepia	54	3·54	2·24	—	100	10·9	—	—	—
Loligo	52	4·35	2·44	6·8	100	3·9	3·5	0·20	0·22
Lithodes	58	2·56	2·60	6·7	100	13·0	3·8	0·12	0·24
Cancer	64	2·44	3·00	3·2	100	13·8	7·0	0·24	0·57
*Carcinus**	62	2·43	2·69	2·4	100	8·0	8·3	0·32	0·68
Palinurus	63	2·05	2·73	2·0	100	10·4	9·2	0·31	0·81
Nephrops	65	1·61	3·02	1·2	100	9·7	11·5	0·43	1·6
Homarus	62	1·75	3·20	1·0	100	4·4	11·5	0·56	2·0

* Calculated from Webb (1940).

In Table 9 are set out the relative ionic composition of the body fluids so far examined, based on a chloride value of 100. Chloride is the most suitable ion to form a basis for the comparison, since it is the ion in the blood closest to the equilibrium value with sea water. Of the twenty animals analysed, all have chloride values within 4% of the sea-water value, most within 1%, the calculations being based, as in all this work, on a mg./g. water basis. The figures represent the gross accumulation and elimination of ions relative to sea water, the data in previous tables representing the net accumulation, in which features such as the Donnan equilibrium and the formation of calcium-protein complexes were allowed for. Several ionic ratios have been calculated on the basis of equivalents. The ratio of Na+K/Ca+Mg remains at the level of that of sea water until we come to the active members of the Crustacea, where it is two to three times higher. The decrease in magnesium is largely responsible for this and also the corresponding increase in the K/Mg and Ca/Mg ratios. Knowledge of the depressant effect of solutions of magnesium salts may induce the speculation that reduction in magnesium has been a factor in the increased activity of some of these crabs and lobsters. The ratio of alkalis to alkaline earths decreases slightly in the Cephalopoda, since its members do not reduce the magnesium content of the blood and have lower sodium values. Indeed, molluscs as a group retain high magnesium levels in their blood. The K/Mg ratio does increase in the cephalopods compared with most of the other molluscs.

Taking the data as a whole, the ionic regulation found in the relatively inactive members of the more primitive morphological classes is slight. The Cephalopoda and pre-eminently the more active of the decapod Crustacea show the most regulation, the latter being the only invertebrate group to lower their blood magnesium significantly. It seems very significant, too, that higher organization and activity are associated with high plasma protein contents, no doubt in part necessary to meet high respiratory demands.

IX. SUMMARY

1. Analyses have been made of the ionic composition of the body fluids of some twenty marine invertebrates belonging to five phyla. The body fluids were again analysed after dialysis in collodion sacs against samples of the original sea water in which the animals had been kept. Comparison of the two analyses in terms of weight of water gives a true measure of ionic regulation by taking into account such factors as the Donnan equilibrium and the formation of calcium-protein complexes in those animals with significant concentrations of protein in their blood.

2. Some ionic regulation is found in all the animals examined, but it is most pronounced in the cephalopod Mollusca and the decapod Crustacea.

3. The mesogloeal tissue fluid of the jelly-fish *Aurelia* showed the following composition (expressed as percentage of concentration in the dialysed fluid): Na 99%, K 106%, Ca 96%, Mg 97%, Cl 104%, SO_4 47%. This regulation seems to be brought about by elimination of sulphate and accumulation of potassium by the epithelia bounding the mesogloea, with resultant alteration in the remaining ions in conformity with osmotic equilibrium between the jelly and sea water.

4. In the echinoderms studied only potassium is regulated, values in the perivisceral fluid not exceeding 111% being found, with higher values in the ambulacral fluid. Polychaetes regulated potassium (up to 126%) and sometimes reduced sulphate (92%).

5. Regulation extends to all ions in the decapod Crustacea. In six species the range was Na 104–113%, K 77–128%, Ca 108–131%, Mg 14–97%, Cl 98–104%, SO_4 32–99%. There is a series *Lithodes*, *Cancer*, *Carcinus*, *Palinurus*, *Nephrops* and *Homarus* in which magnesium falls from 97 to 14%; the series is roughly in accordance with increase of activity. Analyses given of the secretion from the antennary glands emphasize the importance of these organs in controlling the composition of the blood. They eliminate magnesium, sulphate, and sometimes calcium, and conserve the other ions.

6. Lamellibranchs and gastropods accumulate potassium and calcium, and eliminate sulphate to a small degree. Range of values in six species was Na 97–101%, K 107–155%, Ca 103–112%, Mg 97–103%, Cl 99–101%, SO_4 87–102%.

7. Considerable ionic regulation exists in the Cephalopoda, ranges being Na 95–98%, K 152–219%, Ca 94–107%, Mg 102–103%, Cl 101–104%, SO_4 29–81%. In *Eledone* and *Sepia* differential excretion by renal organs is an important factor in this. Sulphate and sodium are eliminated in quantities greater than would be present in an ultrafiltrate of the plasma, tending to lower these values, whereas the other ions are excreted in proportions below those of an ultrafiltrate, tending to elevate their concentrations in the blood.

8. The ratio of equivalents Na + K/Ca + Mg in the body fluids of these marine

invertebrates remains at the sea-water figure of 3·8 in *Aurelia*, echinoderms, annelid worms, and lamellibranchs, but decreases in the gastropods and cephalopods to 3·5. In the decapod Crustacea, owing principally to reduction of magnesium, it increases from 3·8 in *Lithodes* to 9 and 12 in the Palinura and Astacura genera.

I am indebted to the staff of the Marine Station, Millport, particularly to the then Director, the late Mr R. Elmhirst, for the many facilities accorded me during my visits there. Some of the apparatus used in this work was purchased by means of a Royal Society grant from the Parliamentary Grant-in-aid for Scientific Investigations. This paper was written and some of the analyses made during the tenure of a Research Fellowship granted by the Carnegie Trustees, to whom I am grateful.

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THE EFFERENT REGULATION OF THE MUSCLE SPINDLE IN THE FROG

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(*Received* 9 *February* 1949)

(With Eleven Text-figures)

Matthews's experiments (1931 *a*, *b*) have established that the muscle spindle of the frog contains a sense organ which responds to stretch and ceases to respond when the surrounding muscle fibres shorten. In addition, the spindle is known to possess a motor supply of its own, a bundle of small striated 'intrafusal' muscle fibres. These fibres run through the spindle capsule (Barker, 1948; Katz, 1949, fig. 1) where they establish intimate contact with the sensory nerve, while being separated by a lymphatic space from the 'extrafusal' tissue. These muscle fibres are said to lose their striations at the region of contact with the sensory terminals. The anatomical arrangement suggests that the intrafusal muscle bundle pulls directly on the stretch receptor, in contrast with the ordinary muscle fibres which will release the tension on the sense organ if they shorten. A contraction of the intrafusal muscle fibres, therefore, must affect the response of the stretch receptor in a manner very different from that of the surrounding mass of 'extrafusal' fibres. The function of the intrafusal muscle fibres is believed to be a regulation of mechanical 'bias' on the sense organ, but little is known concerning their motor innervation and the physiological conditions under which they are thrown into activity.

Matthews (1931 *b*) obtained evidence in a few experiments which suggested that the intrafusal muscle fibres are innervated by separate motor axons of high threshold and small diameter. More recently, Tasaki & Mizutani (1944), Tasaki & Tsukagoshi (1944) and Kuffler & Gerard (1947) have investigated the properties of small motor nerve fibres which supply many frog muscles and observed that these axons produce a slow local potential change in the muscle which is wholly different in character from the usual propagated muscle spike, and which is accompanied by a slower type of contraction. Kuffler, Laporte & Ransmeier (1947) found that the maximum tetanus tension evoked by the small axons was about 10–15% of the normal twitch tension, and they suggest that the small axon system may be used for the maintenance of peripheral 'tonus'. In view of Matthews's (1931 *b*) work, however, there is an alternative possibility, namely that the atypical muscle responses described by Kuffler and his colleagues arose in the intrafusal motor system, and that their function was to regulate the response of the stretch receptor.

To decide between these two propositions, it is necessary to record the activity of nerve and muscle fibres simultaneously and to study the effect on the afferent discharge of the different types of motor axons. If it be true that the small, high-threshold axons supply the intrafusal muscle fibres, and the large, low-threshold motor axons innervate the rest of the muscle, then one should expect the pattern of response shown in Table 1.

Table 1

	Stimulation of large motor axon	Stimulation of small motor axon
Motor axon spike	Large size, high speed	Small size, low speed
Muscle action potential	Propagated, diphasic spike, large and brief	Non-propagated, mono-phasic, potential, small and long-lasting
Muscle contraction	Large twitch	Weak and relatively slow contraction
Afferent discharge from stretch receptor	Silent period during rise of twitch (i.e. for about 40 msec. at 20° C.)	No silent period but numerous impulses during rise of contraction

Thus, stimulation of the small axons should result in an outburst of sensory impulses, while stimulation of the ordinary motor fibres should be followed by absence of, or a 'gap' in, the afferent discharge (Matthews, 1931*b*).

The results of the present study were contrary to these expectations: the 'silent period' of Matthews was confirmed if the muscle was allowed to shorten freely, but under isometric conditions the ordinary motor impulse was followed by an immediate sensory discharge, and evidence will be presented to show that this was due to the excitation of intrafusal muscle fibres by branches of the large motor axons. On the other hand, small motor axons were found in many cases which elicited the peculiar local responses described by Kuffler & Gerard (1947) without producing sensory discharges. This made it impossible to regard the small axon system as an integral part of the spindle apparatus, although there was evidence in some experiments that branches of the small motor axons may provide an additional nerve supply to the intrafusal muscle fibres.

The conclusions arrived at in this paper are based on physiological evidence. By fine gradation of electric stimuli and of blocking currents applied to the nerve trunk, different types as well as individual motor axons can be separated, and by a simultaneous recording of the electric responses of nerve and muscle, it is possible to trace the intramuscular pathways of the various axons and to analyse their functional connexions. Such an analysis requires verification by histological methods: the anatomical relationship of the nerve endings is being studied by Prof. J. Z. Young and will be described separately.

METHOD

The method of stimulating and recording is shown in Fig. 1. The amplifier was used with condenser coupling, the deflexion to a rectangular input declining to one-half in 0·5 sec. The stimuli consisted of single or double thyratron discharges of short duration (time constant less than 50 μsec.) applied to the sciatic nerve or to the roots of n.spin. IX or X. A constant current was applied through a more distal pair of Ag-AgCl electrodes to block the large axons. The possibility of separating nerve fibres of different size by their different thresholds to stimulating and blocking currents is well known and has been used successfully by others (see Kuffler

& Gerard, 1947). By controlling the intensities of both currents, it was often possible not only to separate the large and small axon groups, but to select individual fibres within each group (see for example Figs. 5, 9, 11 below). In the following the terms 'large' and 'small' motor axons will be used to distinguish between the groups of nerve fibres which are responsible, respectively, for the classical muscle spike and the slow non-propagated response described by Kuffler & Gerard (1947). The distinction between the two types of muscle response is much more striking than that between the size of the nerve fibres concerned, but for convenience the terms 'large' and 'small axon effects' will be retained.

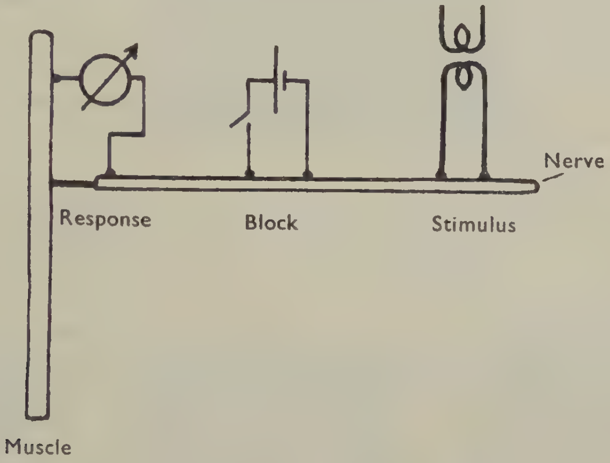


Fig. 1. Arrangement of stimulating, blocking and recording electrodes.

The present method of recording (Fig. 1) allows nerve and muscle action potentials to be seen side by side. There was no ambiguity about the nature of any individual action potential: whether it was a muscle spike or an end-plate potential, an afferent or efferent nerve impulse could be decided from the electric sign of the oscillograph deflexion, from its latency and time course, and when necessary was verified by further checks, for example, by placing both recording leads on nerve or muscle only, or reducing the nerve supply, or stimulating, and recording from, spinal roots. The method of simultaneous nerve and muscle recording was so satisfactory that additional registration of the mechanical response became unnecessary. The weak contraction which followed a single impulse in a small motor axon was observed with a binocular microscope.

The preparation was the M. extensor longus dig. IV of English *Rana temporaria*. The muscle is approximately 15 mm. long and consists of parallel fibres running from end to end. It contains two or three intrafusal bundles, each composed of a number of small fibres along which several spindles are distributed in series (Young, 1949). These structures occupy only a small percentage of the total cross-sectional area of the muscle, most of which is taken up by 50–60 fibres, of diameters varying between 10 and 100 μ (Katz, 1948). The muscle is supplied by about a dozen nerve fibres of which three or four are sensory axons, each contacting one or several muscle spindles. The nerve is a branch of N. peron. lat. which approaches the proximal half of the muscle and divides into two or three twigs before entering it. Occasionally, a sensory twig containing one to three axons leaves the N. peron. lat. at a point a few millimetres proximal to the departure of the main muscle nerve. In some frogs, there was evidence of a few motor axons running in the N. peron. med. and joining the muscle via a connecting branch. In the later experiments, therefore, care was taken to preserve the peroneal nerve plexus, but the results did not differ from those obtained after removal of the medial branch. The preparations were fixed and stained with osmic acid after the experiment, and it was checked whether any nerve fibres had been accidentally cut during the dissection. In some preparations, the nerve supply was intentionally reduced by severing branches until one or two motor and one or two sensory axons remained. In a number of experiments, ventral and dorsal roots of N. spin. IX and X were stimulated separately, and on one occasion, the roots were also used for recording the discharge of afferent impulses from the muscle.

The preparation was mounted in paraffin oil, the tendons of the isolated muscle being gripped by a pair of screw-controlled forceps. In some experiments, one end of the muscle was connected to a light spring so that shortening was permitted with only a slight rise of tension.

When it was desired to apply a curarine solution, a watch-glass was mounted below the muscle, and the preparation was lowered into the solution and soaked until nerve stimuli failed to elicit visible contractions.

The results are based on thirty-eight experiments made during February–September 1948, at temperatures of 13–22° C.

RESULTS

A. *The influence of the large motor axons on the muscle spindle*

(1) *Normal muscle*

If two recording electrodes are placed on the muscle, at the region of the nerve entry and the end respectively, and the nerve is stimulated with shocks of increasing intensity, a threshold is reached at which a large muscle spike is recorded. With further increase of the stimulus strength, the spike remains unaltered until the threshold of another motor unit is reached and a step-like increase in the response occurs. There are usually three or four of these threshold 'steps', all within a twofold to threefold range of shock intensities. If one recording electrode is placed on the nerve, as in Fig. 1, the same muscle spikes are seen, but they are preceded by a brief nerve volley travelling into the muscle, and followed by afferent nerve impulses coming from the muscle. The direction of travel is evident from the sign of the deflexion, the large first phase of the spike being always due to a potential drop at the electrode which is first approached by the impulse.

Fig. 2 illustrates an experiment, at 20° C., in which the muscle was under slight resting tension (about 0·1 g.), its ends being immobilized by pairs of holding forceps. The background discharge of several stretch receptors is seen in the second and third record. When a shock was applied to the nerve, just strong enough to produce a twitch, the shock artifact (S) was followed by: (i) a descending nerve volley (N_1, downward spike); (ii) a large diphasic muscle spike (M, only partly visible); and (iii) a burst of afferent impulses (N_s) beginning at about 7 msec. after the muscle spike. The latency and time course of the twitch which is associated with the spike is well known; according to Sandow (1947), the rising phase lasts about 35 msec. and the total twitch about 150 msec. in a frog's sartorius at 25° C. It is clear, therefore, that the afferent discharge in the experiment of Fig. 2 commenced quite early during the rise of tension, and this appears to contrast with the 'silent period' observed by Matthews (1931*b*). This discrepancy vanishes if the muscle is allowed to shorten. In Fig. 3 the discharge during an isometric twitch is compared with that during free shortening, the resting tension being about 0·1 g. in the latter, and even less in the isometric case. The lower part of the figures shows the initial portion of the response on an expanded time scale. The muscle spike was so large and steep

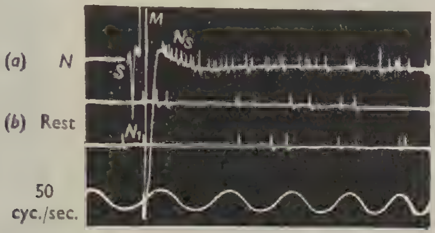


Fig. 2. Electric response of nerve and muscle. In (*a*), a threshold stimulus has been applied to the motor nerve fibres. In (*b*), the afferent discharge from the resting muscle is seen. For further explanation see text. *Note.* The records in all figures of this paper read from left to right, but the direction of the spike deflexion varies in different illustrations.

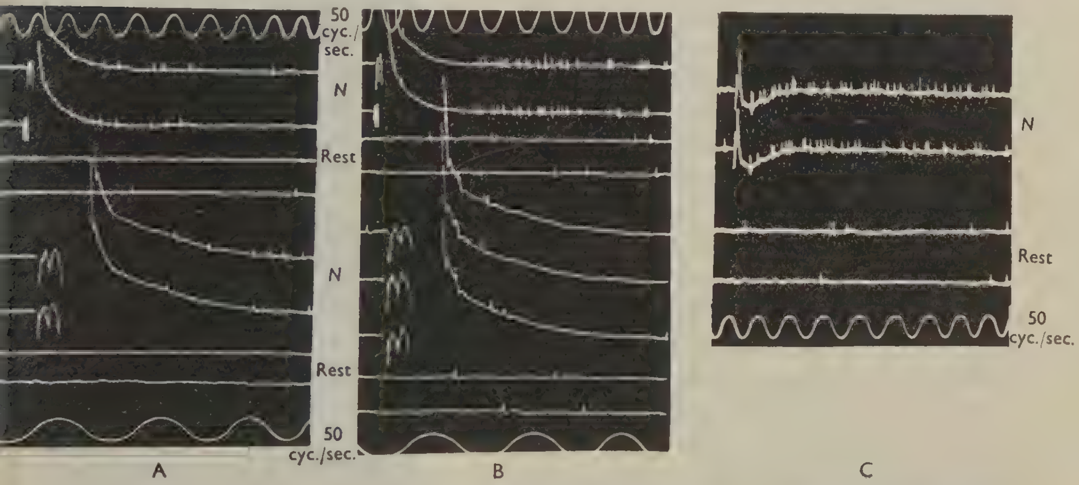


Fig. 3. A and B: responses to nerve stimulation before curarization. C: after curarization (higher amplification). In A, contraction was isometric; in B, shortening was allowed; in C, no mechanical response was seen. See text for further explanation.

that only its slow tail can be seen in the reproduction. During the isometric twitch, there is an afferent discharge commencing at 20 msec. after the muscle spike and lasting 70–80 msec. During the 'isotonic' twitch, there is a distinct silent period lasting about 50 msec. and followed by a burst of impulses during the period of mechanical relaxation (possibly accentuated by an overshoot of the lever). This behaviour is the same as described by Matthews (1931 *b*), except that he observed the silent period even if the ends of the muscle had been immobilized. Presumably, this residual difference is due to the choice, in the present experiments, of a parallel-fibred muscle in which isometric conditions with a minimum amount of 'internal shortening' can be more nearly obtained than in the pennate muscles previously used. This agrees, in fact, with Matthews's own explanation (1931 *b*, p. 162).

It will be noted that the 'silent period' in Fig. 3B is preceded by a few afferent nerve impulses which started as early as 7–8 msec. after the muscle spike. The latency of these impulses is shorter than in Fig. 3A, the difference being attributable to the slightly higher resting tension in Fig. 3B (see following paragraphs).

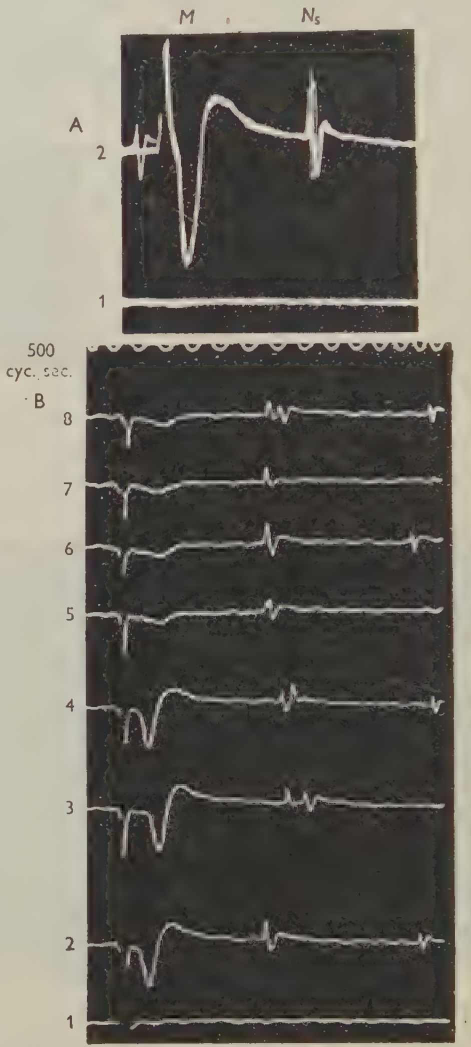


Fig. 4. A, 1 and 2: several sweeps are superimposed in each record, showing the regular appearance of a sensory volley (N_s), about 9 msec. after the muscle spike (M). B: successive records, from 2 to 8, show the persistence of the afferent response during neuro-muscular 'fatigue'.

When the nerve supply had been cut down, an even clearer picture was obtained. In Fig. 4A the preparation contained one motor and two sensory axons. A threshold stimulus to the motor nerve invariably resulted in an afferent volley, 9 msec. after the electric muscle response. Similar observations were made in every experiment; as soon as the stimulus exceeded the threshold of one of the motor units, a muscle spike was set up followed within 7–20 msec. by the firing of afferent impulses. There were considerable variations in the latency and number of these afferent spikes. If the muscle was slack and background activity of the stretch receptors completely absent, the motor impulse sometimes failed to excite them, or only a single afferent impulse might be produced. But in these cases, a slight increase of resting tension sufficed to make the afferent discharges appear after each muscle spike with great regularity. The latency and number of impulses which followed the muscle spike depended evidently upon the background level of excitation at the stretch receptor and could be varied by slight alterations in the resting tension.

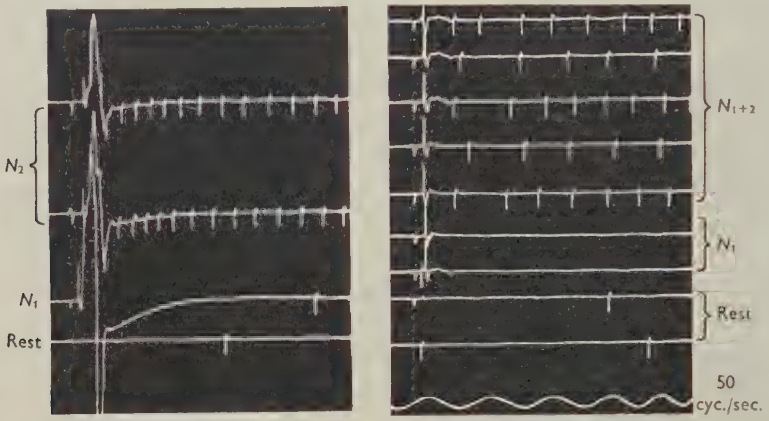
These observations make it seem likely that the afferent firing arises from an activation of the muscle spindle by the motor nerve impulse. The mechanism of this process, however, is still obscure, and even the nature of the afferent spikes is still open to question.

First, it must be proved that the afferent discharges are sensory impulses and originate in the muscle spindles. That they are not repetitive motor impulses coming from the nerve endings is shown: (i) by the absence of repetitive muscle responses; (ii) by the fact that they can be recorded from the dorsal roots of N. spin. IX and X, but not from the ventral roots; and (iii) by their dependence upon resting tension and upon the amount of shortening permitted during contraction. The last two observations (Fig. 3) indicate that the afferent impulses arise in the stretch receptors of the muscle. There are three reasons for classifying these receptors as spindles and not as tendon organs: (i) because crushing the muscle near its tendinous ends did not affect the afferent response; (ii) because several spindles but no tendon end-organ could be demonstrated histologically; and (iii) because the discharge stopped during the period of shortening (Fig. 3 B) which is characteristic of a spindle but contrasts with the behaviour of a tendon receptor (Matthews, 1931 *a*, *b*; 1933).

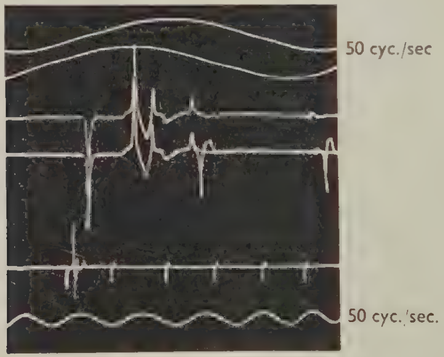
The second question is whether the discharge might have been initiated or influenced by the antidromic impulse which was set up in the sensory axon when the whole nerve was stimulated. In some experiments when a differential block was used, the antidromic impulse did not reach the sense organ so that the present question did not arise. In several experiments, the nerve supply was reduced to one or two sensory axons only, and in these cases stimulation of the sciatic nerve produced a single descending impulse, but no afferent discharge. Finally, in four experiments, the spinal roots were dissected and stimulated separately. In all these experiments, stimulation of the dorsal roots produced one antidromic volley but no afferent firing, while stimulation of the ventral roots produced the same sensory discharge as obtained when the stimulus was applied to the sciatic nerve.

Thus, it is safe to conclude that the afferent impulses from the muscle originate in the spindles and that these are activated in some way by the low-threshold motor axons. The next step was to investigate more closely the relationship between motor nerve impulse and spindle activity.

While the sensory firing was always associated with the excitation of a motor unit, not *every* motor axon produced the effect. In Fig. 5 A, for instance, the sensory nerve supply had been reduced to a single fibre, while the motor supply consisted of four units which could be identified by separate thresholds, spike sizes and velocities and which could be isolated by differential blocking (see Method). The first motor unit produced a large muscle response but no afferent firing until quite late, during the relaxation of the twitch (80 msec. after the muscle spike). This motor axon (N_1) was then blocked and the stimulus increased until the threshold of the next motor unit (N_2) was reached. This unit gave a smaller spike response, but led to an immediate burst of sensory impulses. This is an interesting observation because it shows at once that the afferent firing was not the result of muscle activity as such—the response of the muscle to N_1 was, in fact, stronger than to N_2—but that there are definite relations between the spindles and certain motor axons.



A. Normal B. Curarized



C. Curarized

Fig. 5. Afferent response to impulses in discrete motor axons. The preparation contained axons of well-separated thresholds and conduction velocities, illustrated in C. (Descending nerve spikes give upward, ascending spikes give downward deflexion.) A: a burst of afferent discharges follows an impulse in motor axon N_2, but not in motor axon N_1. B: the same effect after curarization. (N_1 is followed occasionally by a small diphasic muscle spike, but not by afferent discharges.)

(2) *Curarized muscle*

In an attempt to probe further into the nature of this functional connexion, some unexpected evidence was obtained when nerve-muscle transmission was blocked by curarine or fatigue. The important result was that the electrical and mechanical response of the muscle could be drastically reduced without diminishing the afferent discharge.

When the muscle is curarized sufficiently, the descending nerve volley fails to elicit any response, motor or sensory. But in order to obtain this condition, about twice the dose necessary for abolition of a visible twitch is required. With an intermediate dose, the descending impulse still stimulates the muscle spindle, although all visible contractions have disappeared. In the experiment of Fig. 3, for instance, the muscle remained attached to the light 'isotonic' lever while it was immersed in a curarine/Ringer solution. After the twitch had been abolished, the solution was replaced by paraffin oil, and instead of a large diphasic muscle spike of 30 mV., a small monophasic end-plate potential (e.p.p.) of about 0·35 mV. was now recorded. There may have been a small residual muscle spike superimposed on the e.p.p., but its amplitude was certainly not greater than 100 μV. Thus, although the muscle spike had been reduced to less than 0·5 % and the contraction apparently had vanished, the sensory response was undiminished (Fig. 3C). In fact, it was greater than in Fig. 3 B, for the pause in the response associated with the 'extrafusal' shortening had now been filled in with sensory impulses. Even in the curarized preparation it could be shown, by the sudden appearance of an e.p.p., that the sensory discharge occurred when the threshold of a large motor nerve fibre was exceeded.

These observations were confirmed in twenty out of twenty-one experiments in which neuro-muscular transmission was blocked until no mechanical response could be detected. The sensory discharge was sometimes reduced (Fig. 5 B), but in other cases remained unaltered.

Fig. 4 B shows a similar experiment on a 3-axon preparation in which transmission failed because of progressive 'fatigue'. The response in records 2–4 was a propagated muscle spike associated with a twitch, while a little later (records 5–8) the large spike had vanished and only a small potential change remained accompanied by a minute, apparently local, contraction. Yet the afferent discharge persisted without change.

If the dose of curarine was slightly increased a stage of block was attained in which a single motor impulse was ineffective, while two impulses at a short interval succeeded in firing the spindle, still without producing any visible motor response. This is illustrated in Fig. 6: at this critical stage, the response of the sense organ consisted of a whole burst of impulses—or none at all. This in itself is of interest because it indicates that between the arrival of the motor and the discharge of the sensory nerve impulses, there is an intermediary event of an all-or-none character and of a time course much longer than that of the individual nerve spikes.

Some idea of the time course of this event can be obtained by plotting the frequency of the sensory impulses against time, as in Fig. 7, the start of the e.p.p. being taken as the time of origin. The rate of the sensory discharge builds up to a maximum in about 30–40 msec. (19° C.). A similar effect was seen in several other experiments, in some of which the ventral roots were stimulated so as to avoid any influence of the 'anti-dromic' sensory spike. The possible significance of this delayed maximum will be discussed below.

It is clear that impulses in large motor axons can elicit a sensory discharge from the spindle without the intervention of a visible contraction, but it is quite possible that the contraction of one or a few small intrafusal fibres would be concealed by the

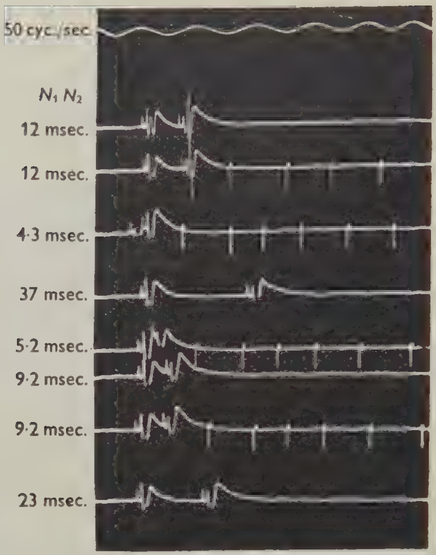


Fig. 6. Facilitation of afferent discharges in curarized muscle. Intervals between nerve stimuli shown in the figure. Note the 'all-or-none' effect at 9·2 and 12 msec.

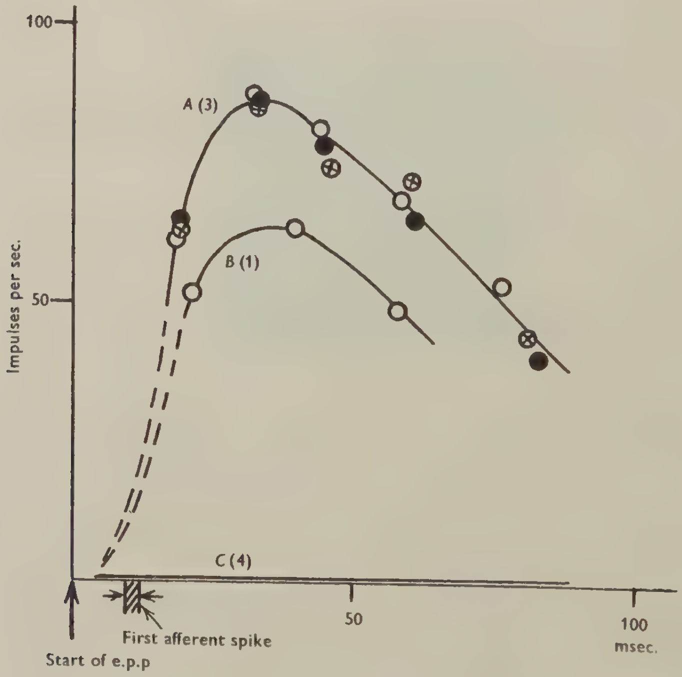


Fig. 7. The 'building-up' effect of the afferent discharge in a critically curarized preparation (same experiment as in Fig. 6). The frequency of the discharge has been plotted against time, taking the start of the second e.p.p. as 'zero time'. The reciprocal of the interval between two impulses has been taken as a measure of the instantaneous rate half-way between them. Out of eight successive records, three followed curve A, one followed curve B and four showed no response (C).

mass of inactive muscle around them, and yet a minute intrafusal twitch would be a very effective stimulus to the spindle receptors. In spite of their apparent complexity all the results would be satisfactorily explained if branches of the ordinary motor axons supplied the intrafusal muscle fibres, and if the intrafusal motor junctions were a little more resistant to blocking than the rest of the muscle.

It is impossible to predict whether or not the mechanical response of the intrafusal fibres should be noticeable. That they can contract there is no reason to doubt, for occasional spontaneous twitches were observed in preparations in which the sensory apparatus and a portion of the intrafusal muscle fibres had been isolated (Katz, 1949). It is possible that the intrafusal bundle is surrounded by a lymphatic space and an extension of the spindle capsule which would allow the fibres to shorten without mechanically interfering with, nor being hindered by, the surrounding muscle. An arrangement of this kind must be postulated if the intrafusal fibres are to pull freely on the sense organ, but it would also make it more difficult to observe a mechanical effect on the whole muscle.

While the mechanical conditions are not clear, it is fairly easy to estimate the electrical response which the intrafusal muscle fibres can give. There are two or three intrafusal bundles in the muscle, each containing several fibres; the diameter of these fibres is of the order of $10\,\mu$, as compared with $50\,\mu$ for the whole muscle (with variations between 10 and $100\,\mu$ (Mayeda, 1890; Katz, 1948)). The total cross-sectional area of the muscle fibres, not including extracellular spaces, is about 10^{-3} cm.^{2} (average of twelve muscles), the intrafusal fibres taking up less than 3 % of this area. The size of the spike would vary approximately with the cross-section of the active bundle, hence the aggregate spike of the intrafusal fibres would be less than 3 % of the maximum muscle spike. In a fairly synchronous maximum volley, the spike of the extensor dig. IV is about 40–50 mV., hence the electric response of all the intrafusal fibres would be about 1 mV., and individual fibre spikes would, on the average, be less than $100\,\mu$V. On the other hand, a large 'extrafusal' fibre of, say, $80\,\mu$ diameter would produce 2·5 mV., i.e. more than all the intrafusal fibres taken together.

In the later experiments, a high amplification was used so that spikes of the order of 50–$100\,\mu$V. should be detected in curarized preparations, even in the presence of e.p.p.'s. The search was not always successful, but in several experiments a minute residual muscle spike could be identified which was always associated with the discharge of sensory impulses. The procedure was as follows. The preparation was curarized until a critical stage of block was reached in which the motor impulse occasionally failed to excite the sense organ. The nerve was stimulated at a rate of about 1 shock per sec. and the oscilloscope was watched for several minutes. The muscle response to successive motor impulses varied in a discrete step-like manner, involving the appearance, at random, of spikes in two or three individual muscle fibres. An example is shown in Fig. 8, which illustrates four distinct patterns of response which recurred in random succession during a large number of exposures. The shock artifact is followed by a descending nerve volley which remained constant throughout the series. The nerve volley (N_1) is succeeded by (*a*) no response, (*b*) a small muscle spike (*m* 0·45 mV.) followed by a few sensory nerve impulses (N_s), (*c*) a large muscle spike (*M* 1·8 mV.) *without* sensory discharge, (*d*) a sum of (*b*) and (*c*). Thus, there was a small muscle spike inseparable from a subsequent firing of the spindle, while the large muscle spike had apparently no connexion with the spindle at all. The small spike may well have arisen in intrafusal muscle fibres, and the large spike in ordinary muscle fibres. The amplitude in this case, viz. 0·45 mV., was greater than in other experiments where an amplitude of 0·1 mV. or less was observed, but it is still compatible with the predicted spike of an intrafusal bundle, assuming that a few adjacent fibres may have fired synchronously. In another experiment, a spike of about 60 μV., which was invariably associated with sensory impulses, was traced in its course along the muscle, and its conduction velocity was found to be 0·64 m./sec. at 17° C. This is about one-third of the highest speed recorded in this muscle (Katz, 1948). According to Offner, Weinberg & Young (1940), conduction velocity in non-medullated fibres, such as muscle, should vary approximately as the square root of the diameter; a ratio of 3 : 1 for impulse velocities corresponds fairly well to the ratio of diameters found for large ordinary (100 μ) and intrafusal (10 μ) fibres.

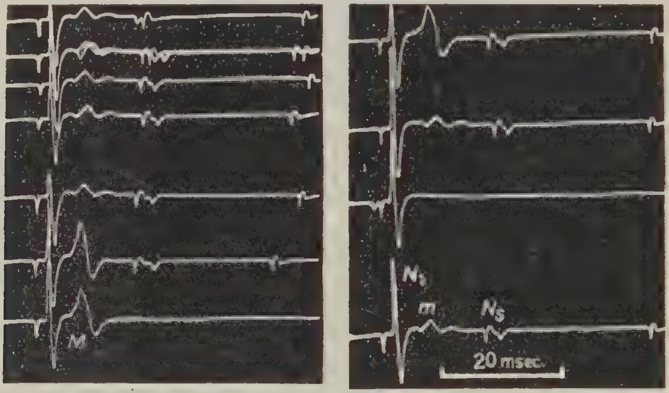


Fig. 8. Evidence of a residual muscle spike (*m*) which is inseparable from a sensory discharge (N_s). For full description see text.

Thus, all the evidence is compatible with the suggestion that the afferent firing is due to a contraction of the intrafusal muscle fibres. The evidence is indirect, but it is strengthened by the fact that the afferent impulses, in critically curarized muscle, occur in groups of an all-or-none character, and that the response builds up to a peak in 30–40 msec. which is approximately the time required for the rise of tension in a twitch (Sandow, 1947).

It was also suggested that the intrafusal fibres are supplied by *branches* of the ordinary motor axons. This was based on the fact that the afferent firing occurred only when a motor unit was thrown into action and that the two responses to a motor nerve impulse, i.e. muscle and spindle response, while not separable by graded stimuli, could be differentiated by progressive curarization. Now, it has frequently been stated that the spindles receive an entirely separate motor nerve supply, and this appears to be borne out by observations on mammalian muscle (Matthews, 1933; Barker, 1948). The situation in frog muscle seems to be a special case, and it is important for a full understanding of the spindle function to obtain definite evidence on this point. It might still be argued that the intrafusal and extrafusal nerve supply, even in the frog, is separate and that (*a*) the motor responses in normal muscle which are associated with a sensory discharge are entirely intrafusal, or (*b*) that separate motor axons of identical threshold supply intrafusal and extrafusal fibres.

The first objection is answered by the fact that in the non-curarized preparation, the size of the muscle responses, which preceded the sensory firing, was usually much too large (spikes of many millivolts and large twitches) to be accounted for by the intrafusal bundles.

The second possibility is more difficult to eliminate, but it could only have been a chance occurrence, and it is impossible to believe that such a coincidence of thresholds could have happened invariably, in more than thirty experiments.

Moreover, in several preparations the nerve supply had been reduced to two or three axons. In these cases it was easy to count the physiological units of response, both motor and sensory, and compare them with the histological count of nerve fibres. In an experiment like that of Fig. 4, where one motor and two sensory axons had been counted on the oscilloscope, histological examination confirmed that there were only three residual medullated axons, and the possibility of separate large motor axons supplying the intrafusal fibres was, therefore, ruled out.

B. *The relation of the small motor axons to the muscle spindle*

The previous experiments dealt only with low-threshold large motor axons which produce in the muscle the classical type of propagated response. There is strong evidence for the suggestion that the intrafusal muscle fibres form part of ordinary motor units, but a part which is relatively resistant to junctional fatigue and curarization. This throws some light on the distribution of motor nerves in frog's muscle, but it is also relevant to the original question concerning the relation between the small motor axons (Tasaki & Mizutani, 1944; Kuffler & Gerard, 1947) and the intrafusal muscle fibres. The small axons might provide an accessory innervation, but they are certainly not the sole supply for the motor apparatus of the spindle. Moreover, the small axons produce weak but visible local contractions in the muscle, and it appears from the experiments described in Section A(2), that even fully propagated activity of intrafusal fibres does not cause a visible movement of the whole muscle.

The question, however, can be tested further by studying the motor and sensory response to stimulation of the small nerve fibres.

By increasing the strength of the stimulus to more than three times the threshold of the ordinary motor axons, the local responses described by Kuffler & Gerard (1947) were obtained. With the help of the constant current block or by microdissection, the small motor axons could be isolated and their effects on the muscle studied in detail. Examples are shown in Figs. 9–10. The results confirm those of Kuffler & Gerard (1947); the potential change in the muscle produced by the high-threshold axons resembles the e.p.p. in its slow monophasic shape and its spatial decrement within a length of a few millimetres. Like the e.p.p. in ordinary curarized motor units, it can sum during successive nerve impulses, is reduced by addition of curarine, and is not lengthened by veratrine. It differs from the e.p.p. in other respects; thus, it gives rise to local contractions but usually not to propagated spikes (cf. Kuffler & Gerard, 1947); its duration is rather larger than that of the e.p.p. (60 msec. as compared with 20 msec., at 20° C.), and the potential recorded at a 'focal' point often shows a very slight positive phase which is not seen with the ordinary e.p.p.

There were frequently three or four small axons of separate threshold, each producing a slow potential change in the muscle. The threshold of these axons varied between 2·5 and 10 times that of the most excitable motor nerve fibre, and the latency of the response, which is largely due to conduction time in the nerve (Kuffler & Gerard, 1947), increased with the threshold.

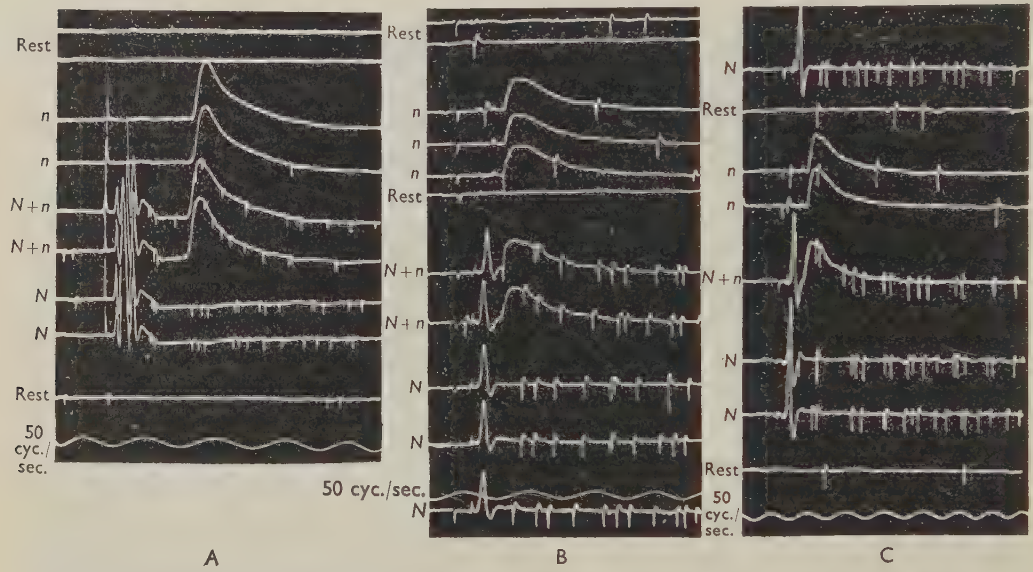


Fig. 9. Separation of small axon responses and afferent nerve spikes. In all three cases, the electric response to a small axon impulse (*n*) consists only of a slow potential change in the muscle, while the response to a large axon (*N*) consists of a rapid muscle spike and afferent firing.

The size of the muscle response was usually somewhat larger than previously described; with a single nerve impulse the potential change was as much as 3–4 mV., and a weak, rather slow, contraction could be seen provided the muscle was slack. This in itself indicates that more than the intrafusal portions of the muscles are involved, for the combined spike of all the intrafusal fibres would have been less than 1 mV.

When the influence of the small motor axons on the spindles was examined, it was found that approximately 50% of the small axon units did not produce any afferent firing, while other units had an effect similar to that previously described (Section A(1)). In eleven out of twenty-one cases, impulses in small axons produced typical local muscle responses without stimulating the stretch receptors. Examples are shown in Fig. 9 which illustrates the sensory background response, the activation of spindles by a large motor fibre, the lack of any additional effect when large and small motor axons were stimulated together, and the absence of any spindle response, in excess of background activity, when the small axon was stimulated alone. Fig. 10 shows an example of a unit which did produce afferent firing. Finally, Fig. 11 illustrates an experiment in which three small axons were identified, of which the first two initiated spindle discharges while the third one did not.

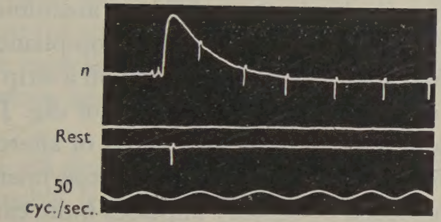


Fig. 10. An example of a small motor axon which produces sensory discharge.

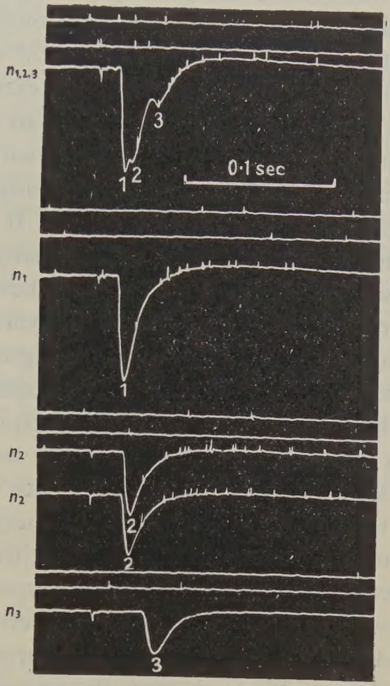


Fig. 11. A preparation containing three small motor axons of which two fire the spindle while the third does not. Records showing combined ($n_{1,2,3}$) and separate (n_1, etc.) impulses in the three axons.

The negative results make it almost certain that the small axon system cannot be a specific part of the spindle mechanism. The original suggestion, viz. that the response to small nerve stimulation might be entirely intrafusal may, therefore, be discarded. The presence of sensory discharges in some cases suggests that branches of small, as well as of large, motor axons can supply the intrafusal muscle fibres, and this would agree with Matthews's (1931 *b*) finding, that in a few preparations the 'silent period' of the spindle was obliterated when the stimulus to the nerve was made very strong.

The real function of the small axon system, and its histological connexions, remain uncertain. The present experiments merely eliminate one possible, and *a priori* quite plausible, role of this system.

DISCUSSION

The main result of these experiments is to show that during active contraction the spindle is subjected to two antagonistic forces: (i) an additional pull exerted by intrafusal fibres which form part of the active motor units; and (ii) a release of tension due to contraction of the 'extrafusal' portions of the muscle. Whether the muscle activity is accompanied by an increase or decrease in the afferent discharge depends upon the balance of these two factors, and this would depend not only upon the external load, but upon the arrangement of the muscle fibres and their physiological condition, fresh or fatigued. It is probable that the type of innervation found in the extensor dig. IV exists in many other frog muscles, in spite of the different pattern of afferent response (Matthews, 1931*b*). It has been suggested that such differences, during 'isometric' contractions, could be due to a different anatomical arrangement of the tendon and a greater or lesser degree of elastic 'compliance' in the active muscle. Preliminary experiments on sartorius muscles and on a strip of M. iliofibularis showed the same sequence of responses as in the extensor dig. IV, i.e. a threshold muscle spike which was followed by an immediate burst of afferent impulses, and there was also evidence in several of Matthews's (1931*b*) experiments that the 'pause' in the spindle discharge is preceded by sensory impulses quite early during the rise of tension, similar to the behaviour illustrated in Fig. 3B. In the present experiments, the spindle response during activity in most of the motor units followed a simple pattern: when there was a mechanical obstacle which the muscle could not move, the afferent discharge was boosted. On the other hand, when the muscle was able to shorten, the sensory discharge stopped. One would expect that any reflex initiated by the proprioceptors of this type of muscle would be enhanced during the ensuing motor action until the mechanical obstacle is overcome. According to Kato (1934, p. 116) the sensory impulses from frog muscle inhibit the excitation of antagonists. Thus, when an extended muscle of the frog is thrown into action, the simultaneous response of its intrafusal fibres would ensure that any excessive resistance of its antagonists is overcome. If the spindle of the frog, like its mammalian counterpart, served the more general purpose of eliciting stretch reflexes, then it will not only start, but maintain the reflex discharge until shortening occurs. Even in the shortened muscle, the spindle tension would not fall very much below the firing level, because the intrafusal bundle has also been thrown into activity.

It is difficult to see why intrafusal motor junctions should be more resistant to fatigue and curarization than the other nerve-muscle junctions. Yet, the striking observations in Section A(2) can hardly be explained in any other way. The matter may depend upon the relative sizes of nerve ending and muscle fibre: perhaps the safety margin in neuro-muscular transmission is greater the smaller the muscle fibre?

SUMMARY

The motor innervation of the muscle spindle of the frog is investigated by recording simultaneously the responses of muscle and nerve when individual axons are stimulated.

There is evidence that intrafusal and ordinary muscle fibres receive a common innervation and that, as a result, the discharge from the spindle is intensified during isometric contraction, but is stopped when shortening occurs.

When the muscle is curarized progressively, a state is reached in which all visible contractions are abolished, yet the spindle continues to respond to a motor nerve impulse with a train of afferent spikes. When the block is intensified, all indirect responses to nerve stimulation fail. Closer study of this effect suggests that the spindle response arises from a twitch of the intrafusal muscle fibres, and that the intrafusal motor junctions are more resistant to curarine than the ordinary junctions.

The existence of an accessory motor system in frog muscle, which is supplied by small axons and characterized by a slow non-propagated response, is confirmed. There is no evidence for any specific connexion between the small motor axons and the muscle spindles, although frequently branches of these axons appear to innervate intrafusal as well as other parts of the muscle.

I am indebted to Prof. J. Z. Young for frequent discussions and to his staff for valuable technical assistance. It is a pleasure to thank Prof. A. V. Hill for the facilities of his laboratory and to Mr J. L. Parkinson for his unfailing help.

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